EFFECT OF *TRANSPARENT TESTA GLABRA1* ON TRICHOME DEVELOPMENT, GROWTH, AND INSECT RESISTANCE IN A *BRASSICA NAPUS AtGLABRA3*$^{+}$ BACKGROUND

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ABSTRACT

Glabrous *Brassica napus* cv Westar and very hairy AtGL3^{+} *B. napus* were transformed using *Agrobacterium tumefaciens* and either a full length trichome regulatory gene *BnTTG1* (isoform 1 coding region called O-TTG1) or an RNAi cassette with 260 bp of a conserved region between isoform I and II (called K-TTG1), each driven by the CaMV 35S promoter. Agronomic and trichome phenotypes were observed in the resulting lines. Transformed lines developed in the glabrous Westar background showed no changes in growth or trichome density and transformation efficiency was similar to that of an empty vector control construct. Over-expression of *BnTTG1* in the AtGL3^{+} *B. napus* background resulted in low transformant survival and poor seed viability, with the only surviving line O-3-7 being taller than non-transformed lines and with a completely glabrous phenotype. The two knock-down lines with the lowest *BnTTG1* expression showed a dramatic increase in trichome density, with longer trichomes and expanded trichome density (up to the 12th leaf in the K-5-8 line) compared to the AtGL3^{+} hairy background line, which showed increased trichome density only on the first three leaves. Moreover, K-5-8 plants were healthy, with both vegetative and reproductive growth similar to that of Westar non-transgenic control plants under both greenhouse and field conditions. The relative expression of five *B. napus* primary trichome regulatory genes and *AtGL3* was measured in three different tissues of *B. napus* Westar, and the AtGL3^{+}, K-5-8 and O-3-7 transgenic lines. Over-expression of *AtGL3* resulted in changes in the expression of *BnGL3, BnGL2* and *BnTRY*. Manipulation of *BnTTG1* levels also resulted in changes in expression of these three genes in addition to *AtGL3*. AtGL3^{+} plants and O-3-7 also showed increased red pigment accumulation in several above ground vegetative tissues including cotyledons, hypocotyl and leaves, whereas the K-5-8 line (knock down of *TTG1*) had less anthocyanin in the same tissues. The level of anthocyanin accumulation corresponded to the relative expression of the three primary anthocyanin regulatory genes *BnDFR, BnANS* and *BnGST*. In a laboratory bioassay, diamondback moth (DBM) adults (*Plutella xylostella*) laid more eggs on hairy leaves of K-5-8 than glabrous Westar. However, more feeding damage from young DBM larvae was observed on Westar leaves than K-5-8 in both choice and no-choice feeding assays. In a field test comparing *Phyllotreta* flea beetle feeding, the hairy K-5-8 leaves showed between a 30-50% reduction in feeding over four ratings on 14 to 28 day old seedlings. Curiously, the glabrous cotyledons of the two hairy lines (AtGL3^{+} and K-5-8) proved to be more resistant than wild type *B. napus* Westar cotyledons from Helix XTra®

insecticide-treated or non-treated seed. These data support the introduction of *AtGL3* and the knockdown of *BnTTG1* to induce a dense trichome phenotype, into otherwise glabrous *B. napus*, resulting in an increased host plant resistance to crucifer insects, without agronomic penalties.

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35S	promoter of the Cauliflower Mosaic Virus 35S protein
µl	micro litre
µm	micro metre
^{0}C	Centigrade
bHLH	basic Helix-Loop-Helix
bp	base pair
CaMV35S	Cauliflower mosaic virus 35S promoter
cDNA	complementary Deoxyribonucleic acid
Ci	Curie
cm	centimetre
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
g	gram
GUS	β-glucuronidase
h	hour
HPLC	High-performance liquid chromatography
kg	kilogram
kV	kilovolt
L	litre
LB	Luria Broth
m	metre
MeOH	Methanol

mg	milligram
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger RNA
MS	Murashige and Skoog medium
MYB	myeloblastosis
NaOCl	sodium hypochlorite
ng	nanogram
PCR	Polymerase Chain Reaction
PNT	Plants with Novel Traits
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNA	Ribonucleic acid
RNAi	RNA interference
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
SSC	saline sodium citrate
TAE	tris-acetate-EDTA
TDNA	transfer DNA
U	unit
UV	ultra violet

CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Brassica napus L., a crop plant cultivated by ancient civilizations in Asia and the Mediterranean belongs to the Cruciferae (Brassicaceae) family (which is also known as the mustard family), was first introduced to Canada in the early 1940's (www.canolacouncil.org, 2012). The family consists of over 40 genera, including more than 200 wild and cultivated species in the US and Canada alone (Callihan *et al*., 2000). The relationship between three diploid species *Brassica rapa*, *B. nigra* and *B. oleracea* forms the "Triangle of U" (**Fig. 1.1**). Hybridization of these three genomes, denoted as A, B and C, respectively, resulted in three amphidiploids *B. juncea* (AB), *B. napus* (AC) and *B. carinata* (BC) (U, 1935). Molecular analysis has confirmed that the amphidiploid genome of *B. napus* is the result of natural crosses between *B. rapa* and *B. oleracea*. RFLP analysis of chloroplast and mitochondrial DNA suggested that *B. montana* (n=9) is the progenitor of *B. rapa* and *B. oleracea* cytoplasms (Erickson *et al*., 1983; Palmer *et al*., 1983). However, different types of organelles and nuclear DNA found in *B. napus* support the concept of multiple origins (Song and Osborn, 1992).

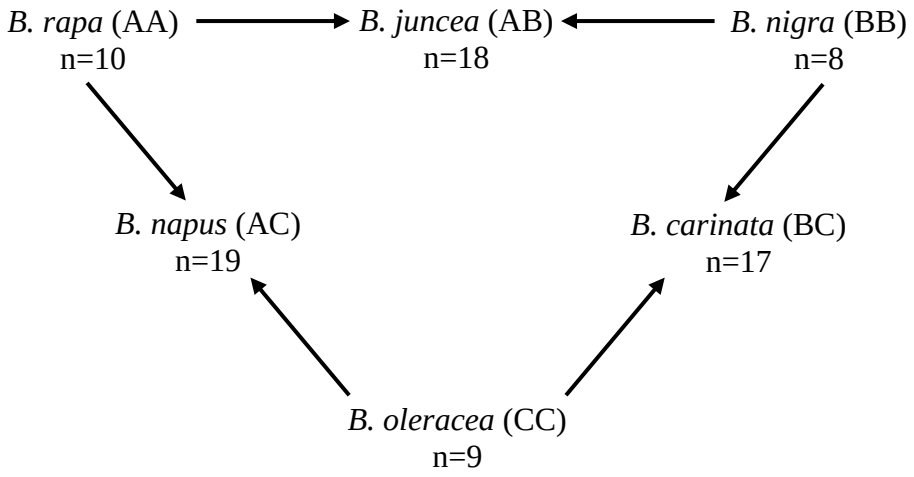


Fig. 1.1. Triangle of U**:** Overview of genetic relationships between cultivated diploid and amphidiploid species of the genus *Brassica* (U, 1935).

Canada's *B. napus* oil seed rape is known as canola and is famous around the world as a nutritious oil seed with low glucosinolate and low erucic acid levels in the seed oil. Canola is Canada's most valuable crop contributing $13 billion annually to the Canadian economy. It is grown throughout Canada but the predominant acreage (18,505,000 acres), is in the Canadian prairies where it is grown annually throughout Saskatchewan, Alberta and Manitoba (www.canolacouncil.org, 2012).

The crucifer flea beetle (*Phyllotreta cruciferae*) is one of the most economically important pests of cruciferous crops on the Prairies, particularly the canola crop. Often appearing in millions, flea beetles (FB) can completely destroy the seedling stage canola (reviewed in Lamb, 1989; Lamb and Turnock, 1982). Canadian farmers lose approximately $200 million annually from FB damage even with an annual chemical application of approximately $40 million to control these pests. While canola crops can also be attacked by a number of other insect pests, including alfalfa looper, beet webworm and diamondback moth (DBM), none are as chronic as FB. Insect control measures must be carefully designed to replace costly and hazardous pesticide applications with their inherent risk of residue buildup in the harvested seed and the environment, resistance buildup in insects, and ability to kill honeybees and native pollinating insects.

Different crucifer plant species possess a range of physical and chemical characteristics that are commonly thought to provide host plant resistance to herbivores. The leaves and seeds of many crucifers, e.g., *Arabidopsis lyrata*, contain a variety of glucosinolates (Clauss *et al*., 2006), a class of natural products characteristic of the family Brassicaceae (reviewed in Jongen, 1996). Trichomes densely cover the leaves and stems and provide a physical barrier to insects for several crucifer species such as *B. villosa* (Palaniswamy and Bodnaryk, 1994), but show only partial coverage in others such as *B. rapa* (Agren and Schemske, 1993, 1994) and *Arabidopsis thaliana* (Arabidopsis) (Mauricio, 1998). In contrast, *B. napus* has very low trichome density and is essentially glabrous (devoid of hair or pubescence). Trichomes in some plant species, such as *Mentha piperita*, accumulate natural products that are insect deterrents, but this has not been shown in the Brassicas (McCaskill *et al*., 1992). A considerable variation in the number of trichomes has been observed in a naturalized population of *B. rapa*, with a range of 1 – 193 trichomes on the right edge of the second leaf (Agren and Schemske, 1994). *Brassica oleracea*, which belongs to tribe Brassiceae, is glabrous (Beilstein *et al*., 2006).

1.2. Development of trichomes in the Brassicaceae

Plant leaf hairs (trichomes) were among the first anatomical features to be recognized by early microscopists. They have been studied intensively and play a key role in plant taxonomy due to their presence on the surface of most plants, their different functions and their morphological variations (Behnke, 1984). In general, trichomes have been defined as unicellular or multicellular structures that originate from the epidermal cell layer of above-ground plant tissues (Fahn, 1974). A glossary of more than 300 descriptions has been used to characterize various morphological types of trichomes (Behnke, 1984), however, they can be defined as two general types: 'glandular-secreting' trichomes (GSTs) with glands that synthesize, accumulate or exude substances, and 'simple' or non-glandular trichomes that do none of the above (Werker, 2000). While non-glandular trichomes are classified according to their morphology, secretory substances are the primary characteristic for glandular trichome classification (Werker, 2000). Since plants can survive without trichomes, many mutations in genes involved in trichome development and branching have been characterized. Several classes of *Arabidopsis thaliana* (Arabidopsis) mutants define specific aspects of non-glandular trichome development, ranging from the specification of the trichome cell to elaboration of its final morphology; a useful resource for understanding cellular differentiation and development at the molecular level (Hulskamp *et al*., 1994; reviewed in Marks, 1997; Schellmann and Hulskamp, 2005).

Epidermal cell-fate specification in Arabidopsis is an excellent example to illustrate the idea of a common pathway guiding different fates in plant development (reviewed in Serna, 2005), and trichome development is a useful model system to study all aspects of cell differentiation (reviewed in Schellmann and Hulskamp, 2005), especially for the Brassicaceae. In Arabidopsis, trichomes are highly specialized single cells that are found on most aerial organs, including the rosette and cauline leaves, on sepals and the stem, but not the hypocotyls, cotyledons, roots, petals, stamens, and carpels (Agren and Schemske 1994; reviewed in Marks, 1997; Schnittger and Hulskamp, 2002; Schellmann and Hulskamp, 2005). Trichomes are especially noticeable on Arabidopsis seedling leaves. The morphology and density of trichomes differ dramatically on different organs; trichomes on rosette leaves have 3-4 branches, cauline leaf trichomes show reduced branching, and stem and sepal trichomes are unbranched (Larkin *et al*., 1996; reviewed in Marks, 1997; Schwab *et al*., 2000; Schellmann and Hulskamp, 2005).

Trichomes originate from specific cell positions in the epidermal layer, with these cells undergoing a sequence of morphological changes, resulting in a mature trichome (**Fig. 1.2**) (reviewed in Schellmann and Hulskamp, 2005). Slightly larger cells with larger nuclei can first be identified within the epidermal layer. By this stage, the nucleus has undergone three rounds of endoreduplication (duplication of the genome without mitosis). The nucleus of this enlarging cell moves to a subapical position with the cell growing out of the surface layer. Incipient branches become apparent when the size of the developing trichome has reached about ten times that of the adjacent epidermal cells. Following another round of endoreduplication, the trichome cell enlarges further by the growth of the stalk and the branches, and the nucleus moves up to the branching point (Hulskamp *et al*., 1994; reviewed in Hulskamp, 2004).

Trichomes start to develop at the distal end of the emerging leaf with the youngest stages located at the proximal end, thereby protecting the more valuable emerging young leaf parts (Woodman and Fernandes, 1991; Larkin *et al*., 1996). A strong degree of uniformity has been observed with respect to the spacing of trichomes on mature Arabidopsis leaves. The frequency of trichomes being initiated immediately adjacent to other trichomes is much lower in Arabidopsis than would be expected by chance (Larkin *et al*., 1996). Also the first 2-3 leaves in the rosette produce trichomes mainly on the adaxial surface, whereas leaves developing later have both adaxial and abaxial trichomes (Telfer *et al*., 1997). Like most Brassicaceae, Arabidopsis does not produce trichomes on cotyledons, although cotyledons of *Sinapis alba* have them on their abaxial surface (Taheri, Nayidu and Gruber, unpublished). Considerable phenotypic variation has been observed in leaf trichome number in a naturalized population of *B. rapa*, and a significant portion of this variation was shown to have a genetic basis (Agren and Schemske, 1993). A wild Mediterranean species, *B. villosa*, has higher trichome densities on both leaf surfaces compared to other *Brassica* species (Palaniswamy and Bodnaryk, 1994).

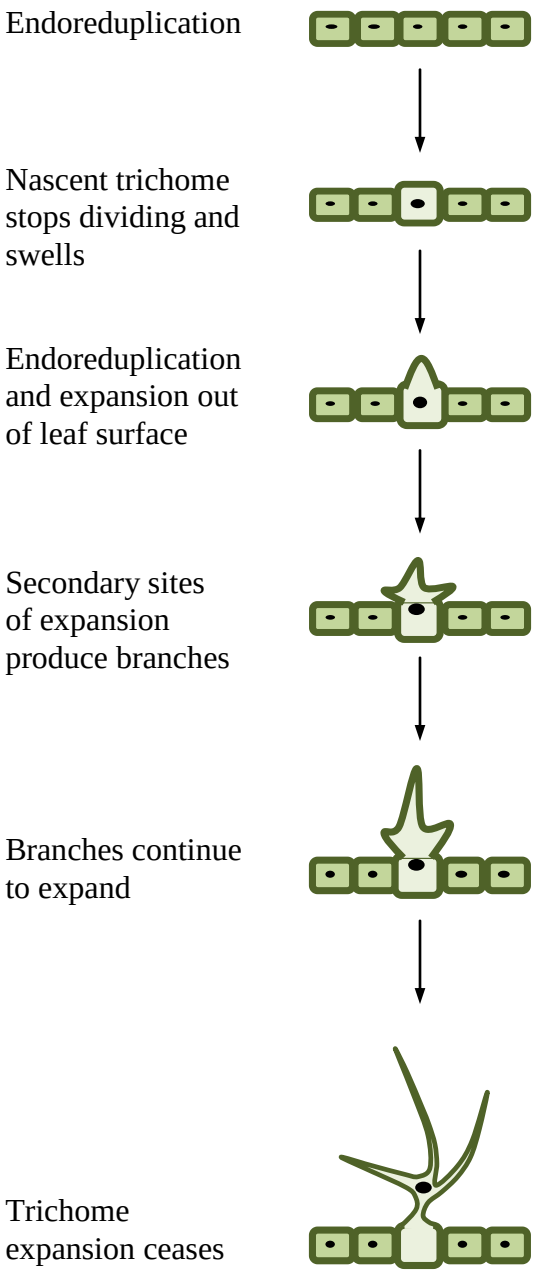


Fig. 1.2. A schematic presentation of branched trichome development.

1.3. Genetic analysis of trichome development

The genetic basis of trichome development in Arabidopsis has been determined to a large extent using a variety of trichome phenotypes that are particularly easy to recognize. Several classes of mutants define specific aspects of trichome development, ranging from the specification of the trichome cell, to branch initiation and elaboration of final morphology. More than 70 genes controlling various aspects of trichome initiation, spacing, size and morphology have been cloned from Arabidopsis (Morohashi and Grotewold, 2009). *TRANSPARENT TESTA GLABRA1* (*TTG1*) (reviewed in Schellmann and Hulskamp, 2005), *GLABRA3* (*GL3*) (Payne *et al*., 2000), *ENHANCER OF GLABRA3* (*EGL3*) (Zhang *et al*., 2003), *GLABRA2* (*GL2*) (Rerie *et al*., 1994), and *GLABROUS1* (*GL1*) (Marks and Feldmann, 1989) (see **Appendix 6.1** for gene family) are all involved in the initiation of trichome development. In contrast, *TRIPTYCHON* (*TRY*) (Schellmann *et al*., 2002), *CAPRICE* (*CPC*) (Wada *et al*., 1997), *TRICHOMELESS1* (*TCL1*) (Wang *et al*., 2007), *TCL2* (Gan *et al*., 2011), *ENHANCER OF TRY AND CPC 1* (*ETC1*) (Kirik *et al*., 2004a), *ETC2* (Kirik *et al*., 2004b) and *CAPRICE-LIKE MYB 3* (*CPL3*) (Tominaga *et al*., 2008) (see **Appendix 6.1**) each contribute to the suppression of trichome initiation. *TRANSPARENT TESTA GLABRA2* (*TTG2*), which encodes a WRKY transcription factor, initially acts downstream of the trichome initiation genes *TTG1* and *GL1* to initiate trichomes (see **Appendix 6.1**). Later in development, *TTG2* shares function with *GL2* in controlling trichome lateral out-growth (Johnson *et al*., 2002). The *35S:AtMYB23* can partially restore the glabrous *gl1* mutant phenotype to the wild-type phenotype and can induce ectopic trichomes on cotyledon surface in Arabidopsis over-expression lines, suggesting that *AtMYB23* has partially overlapping functions with *GL1* in controlling the initiation of trichome development (Kirik *et al*., 2001). *REDUCED TRICHOME NUMBER* (*RTN*) affects the number of trichomes produced per leaf, and consequently increases the distance between trichomes on mature leaves (Larkin *et al*., 1996). Mutations in *LEAFY COTYLEDON1* (*LEC1*) result in trichomes on the adaxial surface of the cotyledons, where trichomes are not normally produced (West *et al*., 1994).

All Arabidopsis trichome mutants, in which the nuclear DNA content is altered, exhibit altered branched trichome phenotypes. Trichome mutants with increased DNA levels such as *try*, *kaktus* (*kak*), *rastafari* (*rfi*), *polychrome* (*pym*) and *spindly* (*spy*) can form up to eight branches, whereas mutants with reduced DNA levels such as *gl3* exhibit only one branch point or are unbranched, suggesting that the level of secondary endoreduplication controls trichome

branching (reviewed in Schwab *et al*., 2000). Mutations in the *SIAMESE* (*SIM*) gene lead to multicellular trichomes. The *SIM* gene suppresses completion of the mitotic divisions resulting in endoreduplication (Walker *et al*., 2000). Mutations in the three genes *ZWICHEL* (*ZWI*), *ANGUSTIFOLIA* (*AN*) and *STACHEL* (*STA*) all result in reduced branching from three to two branches with qualitative differences among mutants. Mature *sta* trichomes have two short branches off a long stalk whereas, *zwi* trichomes have two long branches off a short stalk resembling young wild type trichomes that have yet to undergo secondary branching. Mutations in *AN* result in symmetric trichomes with two long branches (Hulskamp *et al*., 1994). A fourth gene, *STICHEL* (*STI*), regulates branch number in a dosage-dependent manner and its function in cell morphogenesis is not linked to DNA replication (Ilgenfritz *et al*., 2003). Mutations in *TUBULIN FOLDING COFACTOR* (*TFC*) *C* or *TFCA* result in an unbranched trichome phenotype (reviewed in Schellmann and Hulskamp, 2005). The directionality of expansion growth is affected in mutants of eight different genes, which are collectively known as the *DISTORTED* genes (reviewed in Hulskamp, 2004).

1.3.1. Positive primary trichome regulators

In Arabidopsis, *GL1*, *TTG1*, *GL3*, *EGL3* and *GL2* are the key positive regulators of trichome initiation and early morphogenesis (reviewed in Ishida *et al*., 2008). *GL1* was cloned by T-DNA tagging (Herman and Marks, 1989; Marks and Feldmann, 1989) and encodes an R2R3 MYB transcriptional regulator with two MYB repeats comprising its DNA binding domain (Oppenheimer *et al*., 1991). The isolation and analysis of the *TTG1* locus identified TTG1 as a WD-40 repeat protein (Walker *et al*., 1999), while GL2 has sequence similarity to homeo domain proteins (Rerie *et al*., 1994) and *GL3* and *EGL3* encode basic/Helix-Loop-Helix (bHLH) proteins (Payne *et al*., 2000; Zhang *et al*., 2003).

GL1, like other MYB proteins, contains two N terminal repeats of the MYB DNA binding domain (Larkin *et al*., 1993). Mutations in *GL1* result in a complete absence of trichomes and the gene is therefore considered a positive regulator of trichome development (Marks and Feldmann, 1989; reviewed in Schellmann and Hulskamp, 2005). While the highest transcript level for *GL1* was detected in developing trichomes, weak expression was detected in other cells of the leaf, even in adaxial epidermal cells that did not have trichomes. *GL1* was not

expressed in developing stipules, young floral primodia, developing stamens, or developing carpels, all tissues lacking trichomes (Larkin *et al*., 1993).

Over-expression of a *GL1* gDNA from the CaMV 35S promoter in a wild type Arabidopsis background reduced the number of leaf and stem trichomes compared to wild type plants but broadened tissue distribution such that ectopic trichomes were produced on the cotyledons while maintaining a few on the abaxial surface of leaves (Larkin *et al*., 1994). This broadened tissue pattern was enhanced in a *cpc* background (Schellmann *et al*., 2002). Cotyledon trichome production was also enhanced in *35S:GL1* over-expression plants homozygous for *try*, where *try* mutants have glabrous cotyledons (Szymanski and Marks, 1998). High levels of *GL1* transcripts detected throughout the shoot apex of *35S:GL1* plants indicate that the broadened specificity and reduced leaf trichome phenotype did not result from co-suppression at the level of transcript accumulation. *35S:GL1* over-expression in wild type Arabidopsis also reduced endoreduplication levels in trichomes (Szymanski and Marks, 1998). Genes required for the initiation of trichome morphogenesis, such as *GL1*, might also regulate the expression of later-acting trichome branching genes. This would be consistent with the phenotypes of weak *gl1* alleles that not only produce fewer trichomes compared to wild type plants but also incompletely branched trichomes (Hulskamp *et al*., 1994).

GL3 is a bHLH protein controlling trichome initiation, branching and development (Payne *et al*., 2000). The *GL3* gene is moderately expressed in young leaves prior to trichome initiation and is highly expressed in young initiating trichome cells, becoming less or non-detectable in mature trichomes (Zhang *et al*., 2003). In Arabidopsis, GL3 protein can bind to its own promoter to regulate its expression in a negative feedback mechanism (Morohashi *et al*., 2007). Arabidopsis *gl3* plants have trichomes that are smaller and less branched than wild-type trichomes. Although wild type Arabidopsis and most Arabidopsis trichome mutants analysed have 16-fold higher DNA content in their trichome nuclei compared with adjacent diploid epidermal nuclei, *gl3* trichome nuclei show increases of only 8-fold (Hulskamp *et al*., 1994). Over-expression of a *35S:GL3* cDNA in wild type Arabidopsis results in an increased trichome phenotype (Payne *et al*., 2000). Over-expression of *GL1* and *GL3* together in Arabidopsis stimulates a “super-dense” leaf trichome phenotype (20 to 30-fold greater than wild type plants), with a few trichomes additionally present on cotyledons and hypocotyls (Payne *et al*., 2000).

The *TTG1* locus, which regulates several developmental and biochemical pathways in Arabidopsis, encodes a protein of 341 amino acid residues with four WD-40 repeats (Walker *et al*., 1999). *TTG1* transcript is present to some extent in almost all organs of wild type Arabidopsis, including roots, leaves, stems, meristems, flowers, and floral buds, with a high level in floral buds, where there are only a few trichomes on the sepals (Walker *et al*., 1999). In young leaves, *TTG1* is expressed to a high level in incipient trichomes but not at all in more mature leaf parts (Bouyer *et al*., 2008). Loss of function in Arabidopsis mutants of *TTG1* prevents the initiation of most trichomes (Larkin *et al*., 1994). However, clusters of rudimentary trichomes are formed along the leaf margin in weak *ttg1* mutants suggesting an apparent negative role for *TTG1* in trichome initiation (reviewed in Marks, 1997), at least in this tissue.

A *35S:GL1* gDNA construct introduced into a *ttg1* background resulted in clusters of adjacent trichomes on leaves (Larkin *et al*., 1994). From this observation, it was proposed that *TTG1* might have some function in preventing cell division after the cell is already committed to the trichome development pathway (Larkin *et al*., 1994). Over-expression of *GL3* gDNA in wild type Arabidopsis produces an abundance of trichomes on leaves, but not to the same level as in plants lacking TTG1, indicating that a functional *TTG1* is necessary for full *GL3* function (Payne *et al*., 2000). Over-expression of the *GL3* gDNA was a weak suppressor of the *ttg1* trichome defect (Payne *et al*., 2000). Over-expression of *EGL3* and *GL3* gDNA together strongly suppressed the Arabidopsis *ttg1* trichome defect (Zhang *et al*., 2003), while *gl3 egl3* double mutants showed defects in other developmental pathways regulated by *TTG1* (Zhang *et al*., 2003). Over-expression of either the *GL3* cDNA or the genomic clone alone was unable to suppress the *gl3 egl3* mucilage defect in the double mutant but did suppress the anthocyanin defect (Zhang *et al*., 2003). TTG1 physically interacts with both bHLH proteins GL3 and EGL3, through a different domain to that which binds the MYB proteins (Payne *et al*., 2000; Zhang *et al*., 2003). In contrast, GL1 and TTG1 do not directly interact with each other (Payne *et al*., 2000).

In Arabidopsis, EGL3, another bHLH protein is comprised of 596 amino acid residues, making it 39 amino acids shorter than *GL3* (Zhang *et al*., 2003). *EGL3* is moderately expressed in young leaves prior to trichome initiation, with expression being highest in young initiating trichome cells, but neither *GL3* nor *EGL3* expression is obvious in stipules (Zhang *et al*., 2003). In Arabidopsis, e*gl3* mutations give completely hairless plants in the already reduced-trichome

gl3 background. However, *egl3* plants have an unmodified trichome phenotype; therefore *gl3* is epistatic to *egl3*, and mutations at both loci are required to produce a completely glabrous phenotype (Zhang *et al*., 2003). Like the *GL3* genomic clone (Payne *et al*., 2000), the over-expressed *EGL3* genomic clone was a weak suppressor of the *ttg1* trichome defect (Zhang *et al*., 2003). However, unlike *gGL3*, *gEGL3* over-expression suppressed both the anthocyanin and mucilage defects of the *ttg1* mutation (Zhang *et al*., 2003). Co-overexpression of *EGL3* with *GL1* produces supernumerary trichomes on the hypocotyls and cotyledons and excessive trichomes on true leaves (Zhang *et al*., 2003). In the same study, yeast two-hybrid experiments showed that a N terminal fragment of EGL3 interacted with full-length TTG1 and the MYB domains of GL1, CPC and TRY.

GL2 is involved in the morphological development and maturation of Arabidopsis trichomes and encodes a homeo domain protein (Rerie *et al*., 1994). *GL2* is normally expressed in developing trichomes and surrounding epidermal cells of young leaf primordia and developing leaves. *GL2*-dependent GUS staining is also detected in the petiole and in the mid vein of developing leaves. As a trichome reaches its final size, the level of *GL2*:GUS activity increases and remains stable in mature trichomes, for the life time of the cell (Szymanski *et al*., 1998). In both individual *gl1* and *ttg1* plants, no *GL2* expression was detected in any epidermal cells in mature leaves, suggesting that *GL2* expression in fully expanded leaves of wild-type plants is dependent upon the transcription of *GL1* and *TTG1* (Szymanski *et al*., 1998).

The first leaf pair in *gl2* plants initially appears to be virtually glabrous, but later produces aborted trichomes that expand laterally along the leaf surface with only rudimentary trichomes developing on later rosette leaves (Rerie *et al*., 1994). Mutations in *GL2* also affect other development processes, resulting in a lack of seed coat mucilage and ectopic hairs on roots (reviewed in Marks, 1997). The growth and trichome phenotypes of *35S:GL2* over-expression in wild type Arabidopsis ranged widely from wild type, to scarcely viable, to *gl2* mutant-like. However, entopic expression of *GL2* resulted in increased trichome numbers (Ohashi *et al*., 2002).

1.3.2. Negative trichome regulatory genes

To date, a total of seven single-repeat R3 MYB transcription factors have been identified as negatively affecting trichome development, with many of these also being involved in root

hair development. Over-expression of any of the seven negative regulatory genes results in a glabrous phenotype in Arabidopsis, whereas single mutants of each of these genes show different phenotypes (Gan *et al*., 2011). *TRY* encodes a R3 MYB protein lacking the recognizable R2 activation domain and is considered a negative regulator of trichome development (Schellmann *et al*., 2002). A unique phenotype of "nests" of up to four trichomes occurs in adjacent cells in *try* mutants. Trichomes with supernumerary branches were also observed in *try* mutants and the amount of DNA in each trichome cell is increased approximately 2-fold compared with wild type trichomes. The concept of *TRY* as a negative regulator of endoreduplication and trichome development (Hulskamp *et al*., 1994) was confirmed in *35S:TRY* plants which were completely glabrous (Schellmann *et al*., 2002). *TRY* mRNA accumulates in several organs, including roots, leaves, siliques and inflorescences; thus *TRY* is expressed not only in trichome-bearing organs but also in organs lacking trichomes, such as roots and siliques (Schellmann *et al*., 2002). *35S:GL1* over-expressed in *try* plants resulted in a high number of trichomes on cotyledons, a marked increase in the size of trichome clusters, and ectopic trichomes on several organs including the abaxial surface of the first leaf pair, stamens and carpels (Hulskamp *et al*., 1994). In contrast, *35S:GL1* over-expressed in wild type plants resulted in reduced numbers of trichomes on leaves and stems suggesting that *TRY* limits the activity of overexpressed *GL1* (Hulskamp *et al*., 1994; Schnittger *et al*., 1999).

In contrast to the *try* phenotype, only an approximate 2-fold increase in total number of trichomes per leaf was observed in *cpc* mutants compared to wild type, with no effect on trichome endoreduplication (Schellmann *et al*., 2002). However, *cpc* plants also show fewer than normal (wild type) root hairs (Wada *et al*., 1997). While 35S driven over-expression of *CPC* resulted in ectopic root hairs in the roots, the trichome phenotype was the same as that of the *gl2:ttg1* double mutant, i.e. lacking trichomes on leaves, stems, and sepals (Wada *et al*., 1997; 2002). In mature leaves, *CPC* expression is confined to trichomes (Schellmann *et al*., 2002), and it is thought to be the counterpart of *GL1* in roots (Wada *et al*., 1997). The increase in evenly-spaced trichome density in *cpc* mutants suggests that *CPC* acts as a negative regulator of trichome spacing (Schellmann *et al*., 2002). Young leaves show high levels of *CPC* expression in trichomes at all developmental stages and, in addition, ubiquitous expression throughout the young leaf primordium (Schellmann *et al*., 2002). A *GL2*:GUS promoter fusion showed

expression in almost all leaf epidermal cells in the *cpc* mutants, suggesting that *CPC* represses *GL2* expression in wild type Arabidopsis (Wada *et al*., 2002).

Etc1 plants have approximately normal trichome numbers and distribution, and in contrast to *try* there is no detectable effect on trichome branching. Over-expression lines of *ETC1* in a wild-type background lack trichomes on their leaves and produce excessive root hairs. In the shoot, expression of an *ETC1*:GUS promoter fusion results in GUS accumulation in trichome precursor cells and developing trichome cells, an expression pattern that essentially overlaps that of the *TRY* and *CPC* genes (Kirik *et al*., 2004a). In addition to abundant trichomes on the hypocotyl epidermis and cotyledon petioles, the *etc1:try:cpc* triple mutant also produces an unusually large number of trichomes on its leaves (Kirik *et al*., 2004a).

Mutations in *ETC2* have no effect on trichome morphology; however, leaves of *etc2* plants have on average 25% more trichomes than wild type leaves. Similarly, a slight but significant increase in trichome number was observed in *etc2 cpc* double mutants, indicating that *ETC2* may act redundantly with *CPC* during trichome patterning (Kirik *et al*., 2004b). Over-expression of *ETC2* in a wild type background resulted in a decrease in trichome number on all Arabidopsis tissues, with 22 of 42 independent transgenic lines showing a reduced trichome number, while 16 were completely glabrous. *ETC2* expression was found in trichomes of young leaves but not in trichomes of old or fully developed leaves (Kirik *et al*., 2004b).

Arabidopsis plants mutated in *CPL3* have approximately 80% more trichomes but consistently fewer branches than wild-type (Tominaga *et al*., 2008). In contrast to *try*, no clusters are observed in *cpl3* mutants. 35S driven over-expression of *CPL3* resulted in an increased number of root hairs, and no trichomes. *CPL3* expression is stronger in shoots than roots of seedlings, however, it has not been detected in young trichomes (Tominaga *et al*., 2008).

Two negative regulators appear to affect trichome patterning on the inflorescence stem. Loss-of-function mutations in *TCL1* results in a dramatic increase in trichome formation on the inflorescence stem; however, trichome initiation and patterning on rosette leaves is indistinguishable from that of wild-type. *35S:TCL1* transgenic plants show a lack of trichomes (glabrous phenotype) on all organs (Wang *et al*., 2007). Similarly, over-expression of *TCL2* also results in a glabrous plant phenotype, while ectopic trichome formation on inflorescence stems and pedicels were observed in *tcl2* plants (Gan *et al*., 2011).

1.3.3. Updated model for trichome initiation and repression

Arabidopsis protoplast transfection assays have confirmed physical interactions between GL3 and GL1 as well as GL3 and TTG1; however, TTG1 does not directly interact with GL1 (Wang and Chen, 2008). Therefore, GL3 is probably a physical link between the two-cell fate regulators, TTG1 and GL1 (Wang and Chen, 2008). An activator-inhibitor model where the activator activates its own inhibitor has been proposed for these proteins and trichome development in Arabidopsis (**Fig. 1.3**). It has been suggested that a tri-protein MYB/bHLH/WD40 complex consisting of GL1, GL3/EGL3, and TTG1 is an activator which promotes *GL2* expression to activate trichome formation (reviewed in Ishida *et al*., 2008). Expression of *GL2* is significantly increased after 4 hours of *GL3* induction, suggesting that *GL2* is an immediate early direct target of GL3 (Morohashi *et al*., 2007). The activity of GL3 is dependent on the presence of a functional GL1, supporting the model of a GL1-GL3-TTG1 (MBW) complex responsible for activation of *GL2* and subsequent trichome initiation (Morohashi *et al*., 2007). In contrast, expression of *TRY* (Schellmann *et al*., 2002), *CPC* (Wada *et al*., 1997), *TCL1* (Wang *et al*., 2007), *TCL2* (Gan *et al*., 2011), *ETC1* (Kirik *et al*., 2004a), *ETC2* (Kirik *et al*., 2004b) and *CPL3* (Tominaga *et al*., 2008) suppresses trichome initiation in a redundant manner. All seven of these single-repeat MYB proteins can physically interact with GL3 (Gan *et al*., 2011) and some are induced by the MBW complex. These R3 MYB proteins lack the typical transcriptional activation domain and compete with the R2R3 MYB protein GL1 for interaction with the bHLH proteins (GL3/EGL3). Yeast three-hybrid assays have shown that TRY and ETC1 can compete with GL1 for binding to GL3 (Esch *et al*., 2003; Wang *et al*., 2010). *TRY* is expressed in young trichomes (Schellmann *et al*., 2002) and, recently it has been shown that several of the R3 MYB proteins including TRY, CPC and CPL3 can move between cells (reviewed in Pesch and Hulskamp, 2009). These studies give strength to the model that R3 MYB proteins inhibit trichome initiation by disrupting formation of the R2R3MYB-bHLH-TTG1 (GL1-GL3/EGL3-TTG1) complex (**Fig. 1.3**) (reviewed in Ishida *et al*., 2008). Therefore, GL3 titre is one of the most important facets of trichome manipulation, since its over-expression would "soak" up excess TRY (and potentially other inhibitor proteins) in non-trichome cells and allow formation of the GL1-GL3 complex.

Although R3 single MYBs negatively regulate trichome formation through *GL2* by limiting the transcriptional activity of the MBW complex, epistatic analysis of *gl2* and single

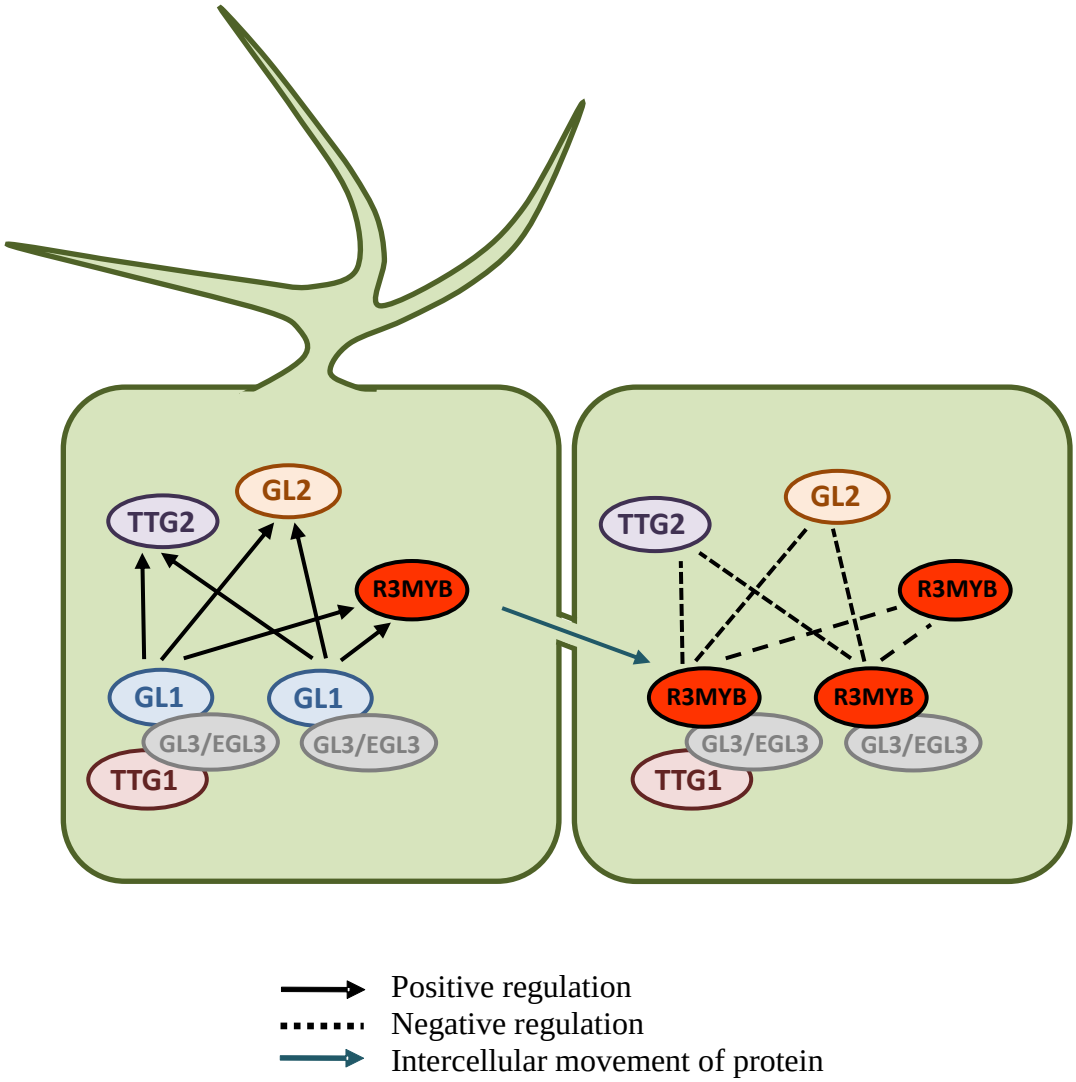


Fig. 1.3. Updated activator/inhibitor regulatory model of di-protein and tri-protein complexes for trichome initiation. The GL1-GL3/EGL3-TTG1 complex promotes *GL2*, *TTG2* and *R3MYB* expression. The R3MYB proteins move into neighbouring cells where they compete with GL1 for binding to GL3/EGL3. Neither the R3MYB-GL3/EGL3-TTG1 complex nor dissociated GL1 can promote *GL2*, *TTG2* or *R3MYB* expression. Cells expressing *GL2* or *TTG2* differentiate into trichome cells (reviewed in Ishida *et al*., 2008). R3MYB represent TRY, CPC, ETC1 and CPL3. Modified from Ishida *et al*., 2007; Wang *et al*., 2008; Wang *et al*., 2010; Gan *et al*., 2011.

myb Arabidopsis mutants suggest that these R3 MYBs may not act simply through *GL2* in regulating trichome formation (Wang *et al*., 2010). TTG2 shares function with GL2 in the trichome development cascade and as with *GL2* expression, *TTG2* expression is also controlled by the MBW complex (Ishida *et al*., 2007) (**Fig. 1.3**).

Di-protein complexes alone also appear to regulate trichome genes in Arabidopsis. Co-transfection of GL3 and GL1 activate the transcription of the *GL2* gene, whereas GL1 or GL3 alone did not (Wang and Chen, 2008). According to the results of Wang *et al*., 2008, a GL1/GL3 combination was as effective as a tri-protein MBW complex in activating the transcription of the single repeat *R3 MYB* genes; however, there was no activation of *TCL1* and *ETC2* by this dual protein complex. Moreover, the expression of *ETC1* was reduced in *gl3 egl3* plants (Wang *et al*., 2008), suggesting that the *ETC1* is tightly regulated by a GL3/EGL3 complex and the expression of *TRY*, *CPC* and *CPL3* was controlled by other mechanisms in addition to a GL1/GL3 complex. Transcription of *TCL1* and *ETC2* are also controlled by unidentified mechanisms (Wang *et al*., 2008).

A second model of activator-depletion has also been proposed for trichome patterning in Arabidopsis (reviewed in Pesch and Hulskamp, 2009). Bouyer *et al*. (2008) found that TTG1 protein is depleted from non-trichome neighbouring cells by accumulation in developing trichome cells. Using microinjections of labelled TTG1 proteins, Bouyer *et al*., (2008) showed that TTG1 protein is moved actively to incipient trichome cells via plasmodesmata by increasing their size exclusion limit. TTG1 is then restrained by GL3 within trichomes (Balkunde *et al*., 2011). Since TTG1 strongly binds to GL3 and this depletion of TTG1 is not observed in a *gl3* mutant, these latter authors suggested that TTG1 is depleted through entrapment in trichome cells by GL3. Moreover, other than restricting the mobility of TTG1, GL3 also mediates the nuclear localization of TTG1 by direct interaction between both proteins (Balkunde *et al*., 2011). As a result of this, cells with more GL3 accumulate more TTG1 and would become more competent to develop into trichome cells whereas neighbouring cells lacking sufficient TTG1 are less competent to become trichome cells (reviewed in Pesch and Hulskamp, 2009).

1.3.3.1. *TTG1* is pleiotrophic

Other than the trichome development pathway, *TTG1* is also involved in Arabidopsis root hair development, accumulation of anthocyanin and seed coat mucilage (Koornneef, 1981).

Mutations in *TTG1* result in an excessive number of root hairs compared to wild type plants indicating that TTG1 is required to prevent the differentiation of immature epidermal cells into hair cells (Galway *et al*., 1994). *Ttg1* plants also lack anthocyanins in the epidermis and subepidermal layers of leaves and stem, and *ttg1* seeds lack the polysaccharide mucilage that normally accumulates on the outer layer of the testa (Koornneef, 1981). However, little has been published on the role of *TTG1* in plant growth (Szymanski *et al*., 1998).

1.3.3.1.1. TTG1 and the relationship between anthocyanin synthesis and trichome production

Anthocyanins are important pigments in the flavonoid pathway and are responsible for a variety of colours ranging from orange to red to purple (Harborne and Grayer, 1988). Biosynthesis of anthocyanins can be influenced by the "signals" released by a plant under stressful conditions (reviewed in Simmonds, 2003). Anthocyanins are important in a variety of functions in the plant. Colour of flowers can facilitate communication between plants and their pollinators, whereas colours of seeds and fruits may aid in seed dispersal by animals and enhance plant reproductive success (reviewed in Schaefer *et al*., 2004). Also, anthocyanins contribute to plant protection against damage by UV radiation and cold temperatures (Burger and Edwards, 1996; Christie *et al*., 1994). Anthocyanins and other flavonoids play roles in host selection of phytophagous insects and are also important in plant resistance to pathogens (reviewed in Simmonds, 2003). The anthocyanin biosynthesis pathway has been studied extensively in plants (**Fig. 1.4**). Genetic and molecular studies aimed at the understanding of (pro) anthocyanin regulation have identified many enzymes and regulatory genes. In particular, research on maize and Arabidopsis describes similarities and differences between the anthocyanin biosynthesis pathways of monocots and dicots, respectively (reviewed in Holton and Cornish, 1995; Petroni and Tonelli, 2011).

Based on altered seed colour, more than 20 genes required for flavonoid biosynthesis in Arabidopsis have been identified (Appelhagen *et al*., 2011). Due to the reduction or absence of pigments in the testa, they are collectively called *transparent testa* (*tt*) mutations (Abrahams *et al*., 2002) although *tannin-deficient seed* (*tds*) mutants also exist. These mutants can be classified into three categories based on the visible pigmentation: mutants producing no visible (pro) anthocyanins (*tt3, tt4, tt5, ttg1*), reduced levels (*tt6, tt7*) or near normal levels (*tt1, tt2, tt8, tt9*,

tt10) (Shirley *et al*., 1995) (**Fig. 1.4**). Similar to the trichome development pathway, involvement of a MYB/bHLH/WD40 tri-protein (MBW) complex has been proposed for the regulation of the flavonoid biosynthetic pathway (Baudry *et al*., 2004). Based on the genes regulated by a tri-protein complex, the flavonoid biosynthetic pathway has been subdivided into early and late biosynthetic genes. Regulation of the late genes is dependent on the tri-protein complex while that of the early genes is not (Gonzalez *et al*., 2008). Early biosynthesis genes are activated by functionally redundant *R2R3 MYB* genes. The MBW complex activates the expression of late biosynthesis genes, leading to the production of proanthocyanidin in seeds and anthocyanins in vegetative tissues. However, the F3'H biosynthetic gene is regulated by both groups of regulators (reviewed in Petroni and Tonelli, 2011).

By interacting with bHLH factors, TTG1 plays a central role in regulating various aspects of secondary metabolism including (pro) anthocyanins in plant tissues, seed mucilage and epidermal cell fate, and linking these pathways to that of trichome development and root hair formation. Expression of the *BANYULS* (*BAN*) gene, which encodes the initial enzyme in the proanthocyanidin-specific part of the flavonoid biosynthesis pathway in Arabidopsis seed coat, can be directly activated by binding of the tri-protein complex TT2/TT8/TTG1 (representing MYB/bHLH/WD40 respectively) to the *BAN* promoter (Baudry *et al*., 2004). Expression of *BAN* is greatly reduced in *tt2, tt8* or *ttg1* mutants. However, over-expression of TT2 or TT8 alone was sufficient for binding to and activation of the promoter and subsequent *BAN* expression in Arabidopsis protoplasts, indicating that the trichome regulator TTG1 may not be necessary for specific recognition of the target DNA by the tri-protein complex. Several other trichome bHLH factors GL3 and EGL3, can also activate *BAN* expression in association with TT2 and TTG1 (Baudry *et al*., 2004). Hence, trichome development and the secondary metabolite flavonoid pathways appeared to be linked through regulatory proteins, in particular TTG1.

In vegetative tissues, the MBW complex consisting of TTG1 and different bHLH and MYB factors regulates anthocyanin accumulation. Over-expression of *MYB113* or *MYB114* resulted in increased anthocyanin accumulation and is TTG1- and bHLH-dependent (Gonzalez *et al*., 2008). A very strong reduction in purple vegetative pigmentation was observed in Arabidopsis plants transformed with RNAi construct targeting the MYB genes *PRODUCTION OF ANTHOCYANIN PIGMENT1* (PAP1), *PAP2*, *MYB113* and *MYB114* (Gonzalez *et al*., 2008). The three *bHLH* genes *GL3*, *EGL3* and *TT8* are functionally redundant in regulating the

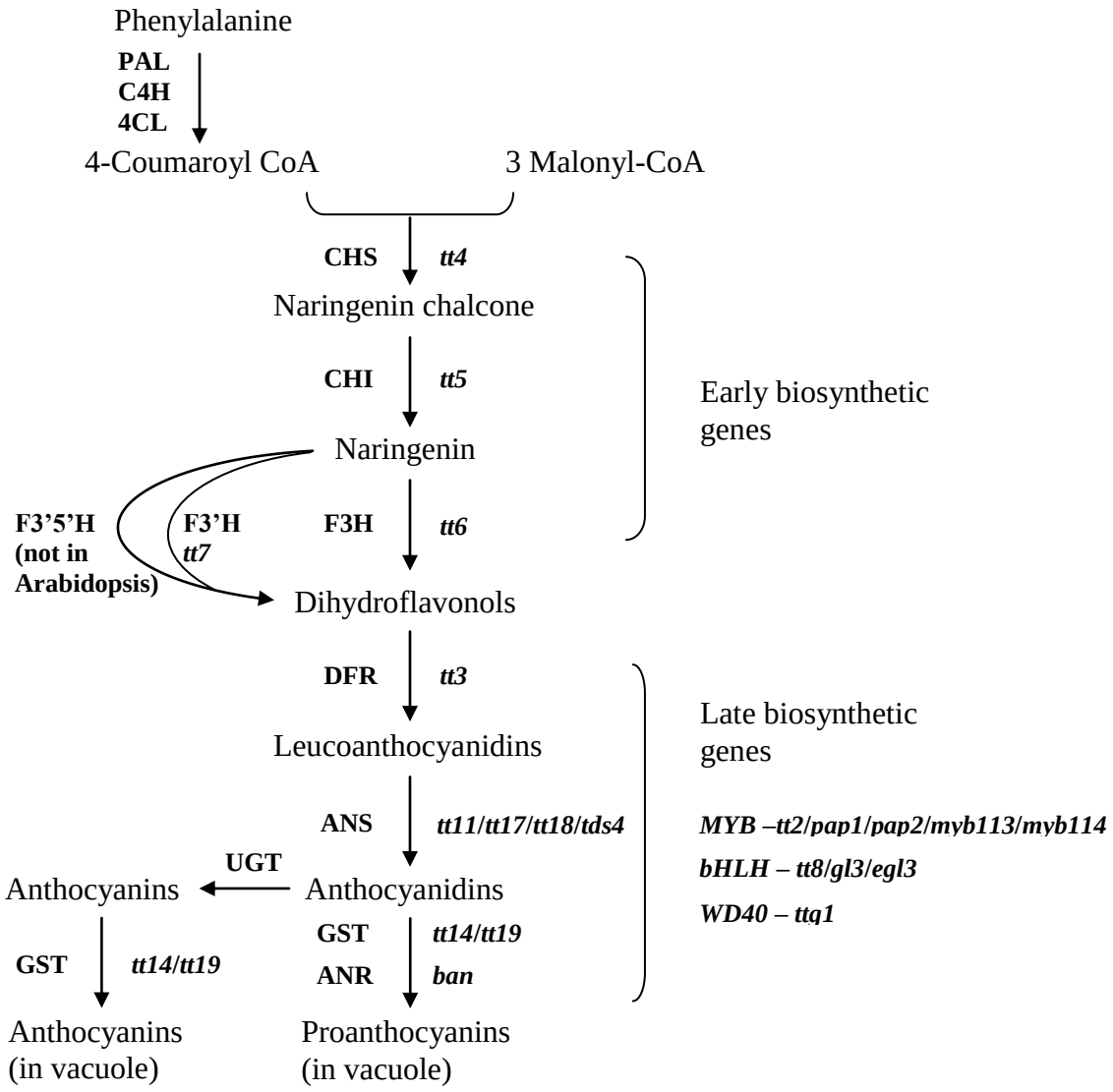


Fig. 1.4. Simplified scheme of the (pro) anthocyanin biosynthesis pathway in Arabidopsis. Enzymes are given in uppercase, the corresponding genetic loci are indicated in lowercase italic letters. PAL, phenylalanine ammonia lyase; C4H, cinnamic acid 4-hydroxylase; 4CL, 4 coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavanone 3′-hydroxylase; DFR, dihydroflavonol reductase; ANS, anthocyanidin synthase; GST, glutathione S-transferase; ANR, anthocyanidin reductase; *pap, production of anthocyanin pigments*; *gl3*, *glabra3*; *egl3, enhancer of glabra3*; *ban*, *banyuls*; *tt, transparent testa*; *ttg1*, *transparent testa glabra1*; *tds, tannin deficient seed*; UGT, glucosyltransferase (adapted from Petroni and Tonelli, 2011; Appelhagen *et al*., 2011).

anthocyanin pathway (Zhang *et al*., 2003). *Gl3* plants look more or less wild-type green in colour, *egl3* plants have reduced anthocyanin in the hypocotyl, whereas *tt8* plants produce a significant amount of anthocyanin in seedling tissue while lacking seed coat proanthocyanidins, indicating that *EGL3* has a more important role in anthocyanin regulation than *GL3* or *TT8* (Zhang *et al*., 2003). In contrast, *GL3* expression is strongly enhanced in vegetative tissues compared with *EGL3* expression when anthocyanin accumulates in response to nitrogen depletion of the growing medium (Feyissa *et al*., 2009). No anthocyanin production was observed in the *gl3 egl3 tt8* triple mutant. PAP1, PAP2, MYB113 and MYB114 all interact with TTG1 and with the bHLH proteins GL3, EGL3 and TT8 (Zimmermann *et al*., 2004).

Two single-repeat MYB proteins, MYBL2 and the trichome/root hair regulatory protein CPC, negatively regulate anthocyanin accumulation by directly inhibiting the activity of the MBW complex on biosynthesis genes (Dubos *et al*., 2008; Zhu *et al*., 2009). *Mybl2* plants increased accumulation of anthocyanin, whereas the over-expression of *MYBL2* in seeds inhibited proanthocyanidin biosynthesis (Dubos *et al*., 2008). Anthocyanin biosynthesis genes are also down-regulated in plants over-expressing the trichome-inhibitor *CPC* gene (Zhu *et al*., 2009).

1.4. The role of trichomes in plant defence

Plants have evolved many defence mechanisms to protect against different abiotic and biotic stresses. Defence mechanisms involving trichomes, in particular, are complex and highly variable.

‘Simple’ or non-glandular trichomes can serve plants in many ways. The morphology and density of trichomes influences several aspects of plant physiology and ecology by mediating the interactions between the plant and its environment (reviewed in Wagner *et al*., 2004). Trichomes play a key role in the water economy of plants, and this is presumed to be one of the adaptive roles of trichomes (reviewed in Johnson, 1975). They directly affect transpiration rates by increasing the thickness of the diffusion boundary layer of leaves, and thus decrease air movement next to the leaf (reviewed in Johnson, 1975). Trichomes also participate in temperature regulation of the transpiring surface by reducing the absorption of radiant energy due to their high reflectance properties, which also protects plant tissue from UV light (reviewed

in Johnson, 1975). Water absorption, seed dispersal and abrasion protection by dead trichomes is also significant (reviewed in Wagner *et al*., 2004).

Since glandular trichomes are major sites of plant natural product synthesis and accumulation, considerable research has been focused on them, including chemical, developmental and transcriptome analysis (Eigenbrode *et al*., 1996; Ascensao *et al*., 1999; Aziz *et al*., 2005; Wang *et al*., 2009; reviewed in Wagner *et al*., 2004). Functions of glandular trichomes can vary in accordance with their location on the plant, the substances they secrete, and the timing of their activity. As with 'simple trichomes', glandular trichomes can be involved in plant temperature regulation and increased light reflectance. Other functions may include ion and pollutant metal secretion and reduced mechanical abrasion (Werker, 2000; reviewed in Wagner *et al*., 2004; Snyder and Antonious, 2009).

1.4.1. Role of trichomes in defense against biotic stress

Plants have evolved different defence mechanisms to cope with pests and pathogens. Since the initial contact point of pests and pathogens is the plant surface, trichomes have evolved as an effective biotic defence mechanism in many plant species. Resistance mechanisms involving trichomes are complex and highly variable. Simple non-glandular trichomes may negatively affect wandering herbivores by physically impeding their movement and feeding. For example, removal of trichomes in *Passiflora lobata* allowed for increased movement of larvae of the non-specialist herbivore, *Heliconius pachinus* (Cardoso, 2008). However, the specialist herbivore, *Heliconius charithonia*, has adaptated to trichomes. Larvae are capable of freeing themselves from entrapment by eating trichome tips or by laying silk mats on trichomes (Cardoso, 2008).

The role of non-glandular trichomes as insect deterrents in the Brassicaceae has been studied in some detail. In North Carolina, the most common herbivores collected on *Arabidopsis thaliana* are two types of FBs, *Psylliodes convexior* LeConte and *Phyllotreta zimmermani* Croth (Chrysomelidae) (Mauricio, 1998). When the plants were exposed to a natural assemblage of herbivores and pathogens, *A. thaliana* lines with higher levels of both trichome density and total glucosinolate concentration experienced less leaf damage than lines families with lower levels of these two characteristics (Mauricio, 1998). Mature leaves of *Brassica villosa* are extremely pubescent and are highly resistant to FB feeding, while *B. rupestris*, *B. macrocarpa* and *B. napus*

with few trichomes suffer significant damage from *Phyllotreta* FB feeding (Palaniswamy and Bodnaryk, 1994). When *B. rapa* lines with high and low trichome densities were exposed to larvae of *Pieris rapae*, the cabbage seed butterfly, low-density trichome plants were preferred by the larvae, which consumed twice the leaf area, compared to high trichome plants. Low-density trichome plants also experienced three-fold greater floral damage compared with high-density trichome plants (Agren and Schemske, 1993). Pods of *Sinapis alba* covered with large numbers of trichomes also showed much greater resistance to FB feeding than those of *B. napus* lacking trichomes, whereas removal of hairs from the *S. alba* pods resulted in an increase in FB feeding damage (Lamb, 1980).

Although trichomes themselves are not widely considered as entry points for pathogens, there is evidence that damaged trichomes can serve as infection sites (Charles *et al*., 2008). Van de graaf *et al*. (2002) found that germ tubes of the *Phoma clematidina*, which cause clematis wilt, regularly entered the plant through glandular and non-glandular trichomes. However, trichomes can also be a source of resistance to plant pathogens. A positive correlation between trichome density and resistance to insects is important in controlling arthropod-vectored diseases (Gunasinghe *et al*., 1988; Ren *et al*., 2000). Secretion of antimicrobial biochemicals to the phylloplane usually synthesized by glandular secreting trichomes (Campbell *et al*., 1980) is another defensive strategy used by some plants to deter potential pathogens (Shepherd *et al*., 2005; Nonomura *et al*., 2009; Luo *et al*., 2010).

Impacts on plant yield have to be considered in plant improvement programs involving the trichome resistance trait. While several experiments have been conducted to measure the physiological costs of trichome production on yield in hairy *B. rapa*, a negative impact in terms of reduced survival or number of flowers has not been detected (Agren and Schemske, 1993). In fact, *B. rapa* plants with higher trichome densities were found to produce significantly more seed compared to plants with low trichome densities (Agren and Schemske, 1994). However, Elle *et al*. (1999) argued that deficiencies existed in either the system chosen or the experimental design in these latter experiments.

During the process of crop domestication, many of the potent resistant traits of the wild progenitors have been lost. These losses have resulted in an increased susceptibility of domesticated crops to insect pests and pathogens. Reintroduction of these traits through

conventional breeding programs or molecular approaches is essential for future plant improvement and for the development of stable resistance to insect pests and pathogens. This is especially important, as an increasing number of pests and pathogens are becoming resistant to synthetic chemicals.

Understanding the molecular biology behind trichome development would be highly beneficial to the glabrous oil seed crop species *B. napus*, since it could enable us to modify trichome densities and architecture and provide stable resistance to insect pests. Arabidopsis, as a close relative to *B. napus* (Parkin *et al*., 2005), is an excellent model system to assist in a study of the molecular biology of trichome development in the Brassicas. A minimum of 21 conserved blocks, which have been replicated and rearranged to generate the present day *B.napus* genome, have been identified within the Arabidopsis genome (Parkin *et al*., 2005). Transformation of *B. napus* cv Westar with *35S:AtGL3* construct has already produced an extremely dense covering of trichomes on seedling tissues (Gruber *et al*., 2006). These "Hairy Canola" lines show strong feeding deterrence to the crucifer FB (Soroka *et al*., 2011), as well as modest resistance to larval feeding of DBM, another economically important pest of canola (Adamson, 2008). However, very little work has been carried out on the molecular basis of trichome development either in the glabrous oil seed crop species *B. napus* or on any other Brassicas in the Triangle of U (U, 1935).

1.5. Hypothesis

Changes in the expression of *TTG1* in *B. napus* can modify trichome development by affecting the relative expression of primary trichome development regulatory gene (s), and the resulting phenotype will provide improved host plant resistance to insect pests.

1.6. Objectives

1. Determine the role of *B. napus TTG1* in trichome development in *B. napus*.
2. Determine the role of *B. napus TTG1* in anthocyanin accumulation and seed mucilage production in glabrous *B. napus* cv Westar and in *hairy* $AtGL3^{+}$ transgenic *B. napus*.
3. Carry out field evaluations of the agronomic characteristics of the new *hairy* K-5-8 transgenic *B. napus* line.
4. Determine the role of trichomes in resistance to crucifer herbivores.

CHAPTER 2. THE FUNCTION OF THE *TRANSPARENT TESTA GLABRA1* GENE ON TRICHOME DEVELOPMENT, GROWTH, ANTHOCYANIN ACCUMULATION AND SEED MUCILAGE PRODUCTION IN *BRASSICA NAPUS*

Glabrous *B. napus* cv Westar and very hairy AtGL3$^+$ *B. napus* were transformed, using Agrobacterium mediated gene transformation, with either the full length trichome regulatory gene *BnTTG1* isoform 1 coding region (O-TTG1) or an RNAi cassette of 260 bp of a conserved region between isoform I and II (K-TTG1) driven by the CaMV 35S promoter. Agronomic traits and trichome phenotypes were observed in the resulting lines. Survival for O-TTG1 transformants in the AtGL3$^+$ background was very low, such that only one line (O-3-7) was developed to homozygosity. Two "knock-down" K-TTG1 lines (K-5-8 and K-6-3) in the AtGL3$^+$ *B. napus* background were developed into single TDNA insertion homozygous lines. Compared to glabrous Westar control plants, leaves and stems were completely glabrous in O-3-7 whereas K-5-8 and K-6-3 showed a dramatic increase in trichome density. K-5-8, with the lowest *BnTTG1* expression, showed the highest trichome density with trichomes continuing to develop up to the 11th or 12th leaf stage. In contrast to the AtGL3$^+$ plants, K-5-8 plants were healthy and growth was similar to Westar non-transgenic control plants under greenhouse conditions. *BnTTG1* over- and knocked-down expression in the Westar background did not show any phenotypic differences with respect to growth or trichome density. The relative expression of five *B. napus* primary trichome regulatory genes and *AtGL3* was measured in three different tissues of *B. napus* cv Westar, AtGL3$^+$, K-5-8 and O-3-7 relative to their expression in *B. napus* cv Westar cotyledons. Over-expression of AtGL3 resulted in changes in the expression of *BnGL3*, *BnGL2* and *BnTRY*. Manipulation of *BnTTG1* levels also resulted in changes in the expression of these three genes in addition to *AtGL3*. AtGL3$^+$ plants showed an increased anthocyanin accumulation in above-ground vegetative tissues whereas the knock-down of *TTG1*, K-5-8 line had less anthocyanin in the same tissues. The level of anthocyanin accumulation corresponded to the relative expression of the three primary anthocyanin regulatory genes *BnDFR*, *BnANS* and *BnGST* in each line.

2.1. Introduction

In Arabidopsis, the mutant *transparent testa glabra1*, mapped to chromosome 5 (Koornneef *et al*., 1983), lacks seedling trichomes, anthocyanins, seed proanthocyanidins,

mucilage on the surface of seeds, has an altered seed-coat morphology (Koornneef, 1981), and produces excessive numbers of root hairs (Galway *et al*., 1994). The *TTG1* gene involved in the regulation of several of these developmental and biochemical pathways, was isolated by positional cloning of this locus and encodes a protein that contains WD40 repeats (Walker *et al*., 1999). WD40 repeat proteins, found in all eukaryotes but not in prokaryotes, are involved in a variety of functions such as cell division, gene transcription and transmembrane signalling (Neer *et al*., 1994). TTG1, in Arabidopsis, is part of a tri-protein complex with the bHLH protein GLABRA3 and the MYB protein GLABROUS1, and several lines of evidence suggest that this complex is involved in the induction of the *GLABRA2* gene and subsequent trichome initiation (Morohashi *et al*., 2007).

Loss-of-function alleles of *TTG1* prevent initiation of most trichomes in Arabidopsis (Larkin *et al*., 1994), however, over-expression of *TTG1* does not result in increased trichome numbers (Payne *et al*., 2000). The weak *ttg1-10* allele with a reduced *ttg1* mRNA level without an altered protein sequence, has trichome clusters along leaf margins, while also exhibiting normal seedling pigmentation and seed mucilage (Larkin *et al*., 1999). However, this allele does result in a lack of testa pigmentation (reviewed in Marks, 1997). A few trichomes and occasional trichome clusters were also observed on the *ttg1-13* null mutant, with the clustered trichomes originating from adjacent protodermal cells (Larkin *et al*., 1994, 1999). These data suggest that, in addition to its positive role in trichome initiation, TTG1 may also play a role in the lateral inhibition of trichome development in neighboring cells.

TTG1 is expressed in all major organs of Arabidopsis and the TTG1 protein is present in all stages of leaf and trichome development (Zhao et al., 2008). *GL2*, *ETC1*, *CPC* (Morohashi et al., 2007) and *TTG2* (Zhao et al., 2008) have been identified as direct targets of TTG1 and GL3, indicating that these two proteins share many of the same targets in the Arabidopsis trichome development pathway (Zhao et al., 2008). Over-expression of *GL3* restores trichome, anthocyanin and seed coat mucilage production in a strong *ttg1-1* mutant background (Payne *et al*., 2000). Abundance of trichomes are produced, however, when the *GL3* gene is over-expressed in an Arabidopsis wild-type background, indicating that TTG1 is necessary for full function of GL3 (Payne *et al*., 2000). Similarly, over-expression of the *bHLH EGL3* gene was able to weakly restore trichomes in the *ttg1* background as well as also restoring wild type mucilage and transparent testa phenotypes (Zhang *et al*., 2003). However, together in *GL3/EGL3*

over-expression lines in the *ttg1* background, these two genes are extremely strong suppressors of the *ttg1* trichome defect (Zhang *et al*., 2003).

Several lines of evidence indicate broad, somewhat complex, TTG1-dependent and TTG1-independent pathways in Arabidopsis. The TTG1 protein directly interacts with several bHLH proteins that directly interact with sets of MYB proteins to stimulate the down stream elements in TTG1 dependent pathways (Zhang *et al*., 2003). In contrast, the expression of EGL3 in non-hair cells of an Arabidopsis root is negatively regulated by TTG1 (Bernhardt *et al*., 2005). However, over-expression of *GL1* (*MYB*) and maize *R* or Arabidopsis *GL3* (*bHLH*) together can bypass the requirement of *TTG1* in trichome initiation (Szymanski *et al*., 1998; Payne *et al*., 2000). These findings support both TTG1-dependent and TTG1-independent control of MYB and bHLH proteins in trichome development.

Two functional homologues of the Arabidopsis *TTG1* gene have been identified in cotton, with both of these genes (*GhTTG1* and *GhTTG3*) able to restore wild type trichome phenotype when introduced into Arabidopsis *ttg1* mutants (Humphries *et al*., 2005). Two Brassica *TTG1* homologs have been identified from hairless, yellow-seeded and hairy, black-seeded *B. rapa* lines (Zhang *et al*., 2009). The amino acid sequence in the hairless, yellow-seeded line produces a non-functional truncated protein, whereas the amino acid sequence in hairy, black-seeded *B. rapa* lines shows 93% identity to the Arabidopsis *TTG1*. Complementation of an Arabidopsis *ttg1* mutant with the *BrTTG1* ortholog from the hairy, black-seeded line rescued both wild type seed colour and wild type hairy phenotype, suggesting that this ortholog functions in a similar manner to that of Arabidopsis *TTG1* (Zhang *et al*., 2009). However, no phenotypic changes were produced with the *BrTTG1* ortholog from hairless, yellow seeded lines. A WD40 repeat protein closely related to Arabidopsis TTG1 has also been isolated from pomegranate fruit and was able to recover the wild type trichome, anthocyanin and seed coat mucilage phenotypes when introduced into an Arabidopsis *ttg1* mutant (Ben-Simhon *et al*., 2011).

With limited information in these *B. rapa*, cotton, and pomegranate studies on the relationship between *TTG1* expression level and gene dosage on full complementation of *ttg1* phenotypes, or effects on *GL3* transcript levels, a study was initiated on the effect of variable dosage of *TTG1* in *B. napus*. Here, the role of *TTG1* in trichome development, anthocyanin

accumulation, seed coat mucilage production and plant growth were examined by over-expression or knock-down of the *B. napus TTG1* in the almost glabrous *B. napus* cv Westar background and in the very hairy AtGL3^{+} *B. napus* background (Gruber *et al*., 2006). The transcript levels of *BnGL1*, *BnGL2*, *BnGL3*, *BnTTG1*, *BnTRY* and *AtGL3* were also characterized in these over-expression and knocked-down lines.

2.2. Materials and methods

2.2.1. Plant material and growth conditions

B. napus cv Westar and homozygous AtGL3^{+} hairy *B. napus*, resulting from an over-expression of the *AtGL3* gene under the control of CaMV 35S promoter in a Westar background, were used. *Brassica napus* was cultivated in CoCo mix (see **Appendix 6.4**) in 15 cm pots and grown in a greenhouse with a 22/18^{0}C temperature regime and a 16/8 h light/dark photoperiod (230 µE.m^{-2}.s^{-1}). Pots were covered with clear plastic flats until seeds germinated and small plantlets were acclimated to greenhouse conditions. When plants started flowering, they were wrapped individually with perforated pollination bags (Cryovac Sealed Air, Canada) and gently shaken by hand once every two days to promote self-pollination. Mature seed pods were collected into paper bags which were stored at 4^{0}C.

2.2.2. Constructs

Molecular cloning was conducted following standard lab procedures (Sambrook *et al*., 1989). Cloning products were verified by automated sequencing (National Research Council – Plant Biotechnology Institute (NRC–PBI), Saskatoon, SK). *Escherichia coli* strain DH10β and *Agrobacterium tumefaciens* GV3101 were used as bacterial hosts for all transgene and empty vector controls.

2.2.2.1. *TTG1* over-expression

The full-length Bn*TTG1* isoform 1 coding region was amplified using PCR specific primers (**Table 2.1.** OFttg1 and ORttg1, see **Appendix 6.2** primer coverage) from first-strand cDNA synthesized using SuperScript™ II RT (Invitrogen, California, USA). The PCR product was purified using a QIAquick® PCR purification kit (QIAGEN, Hilden, Germany), cloned into pGEM®-T Easy Vector System (Promega, Wisconsin, USA) and transferred into *E. coli*. Ten positive colonies were sequenced. Plasmids containing the perfect insert sequence match to the

Table 2.1. Primer sequences used in the construction of *BnTTG1* vectors, screening of transformants and genome walking analysis. * Restriction enzyme sites are underlined.

Name	Function	Primer sequence (5' to 3')	Fragment size (bp)
OligodT	cDNA synthesis	TTTTTTTTTTTTTTTTT	-
35S-F	Screening of transgenic plants	TTGGAGAGAACACGGGGGAC	-
OFttg1	O-TTG1 construct	CGGGATCCATGGACAACTCAGCTCCAGA*	1014
ORttg1		CGGAGCTCTCAAACTCTAAGGAGCTGCA*	
LFttg1	K-TTG1 construct	CGTCTAGATAGGCACGTGCAGCATCGAC*	260
LRttg1		CGGGATCCACACCTCAAGTCCTGCTTGT*	
RFttg1	K-TTG1 construct	CGGAGCTCTAGGCACGTGCAGCATCGAC*	260
RRttg1		CGTACGTAACACCTCAAGTCCTGCTTGT*	
FBAR	Screening of transgenic plants	AAGTCCAGCTGCCAGAAAC	438
RBAR		CACCATCGTCAACCACTACA	
FGUS	Screening of transgenic plants	ATGTTACGTCCTGTAGAAACCC	384
RGUS		TACACTTTTCCCGGCAATAACA	
AP1	Genome walking	GTAATACGACTCACTATAGGGC	-
AP2		ACTATAGGGCACGCGTGGT	
GW-BAR	Genome walking	GCCCCGTCCGGTCCTGCCCG	-
GW-BAR'		GCCCGTCACCGAGATCTGATGA	

transgene were extracted using a QIAprep® Spin Miniprep Kit (QIAGEN), digested, and the transgene cloned into the *BamH*I/*Sac*I sites of the binary vector p79-103 (Agriculture and Agri-Food Canada, Saskatoon Research Centre). *A. tumefaciens* GV3101 was transformed with the *TTG1* transgene over-expression construct O-TTG1 or p79-103 empty vector control construct (**Fig. 2.1A**).

2.2.2.2. *TTG1* knock-down

For silencing of *BnTTG1*, a 260 bp conserved region between isoform I and II were chosen as the target sequence and amplified using PCR specific primers (**Table 2.1.** LFttg1/LRttg1 and RFttg1/RRttg1 respectively, see **Appendix 6.2** primer coverage) from first-strand cDNA synthesized using SuperScript™ II RT. The PCR products were purified using a QIAquick® PCR purification kit, cloned into the pGEM®-T Easy Vector System and transferred into *E. coli*. Ten positive colonies for each PCR product were sequenced. Plasmids with a perfect sequence match to the transgene were extracted using the QIAprep® Spin Miniprep Kit, digested, and two identical *TTG1* fragments were cloned in sense and antisense orientation into the GUS intron-containing intermediate vector pBI121 (Clontech, California, USA) at unique *Xba*I/*BamH*I and *Snab*I/*Sac*I sites. The resulting sense-intron-antisense cassette was subcloned into the unique *Xba*I/*Sac*I sites of the binary vector p79-103 (as above). *A. tumefaciens* GV3101 was transformed with the sense-intron-antisense final binary construct K-TTG1 or a p79-103 empty vector control construct (**Fig. 2.1B**).

2.2.3. Development of stable transgenic plants with modified *TTG1* expression

2.2.3.1. Agrobacterium-mediated plant transformation

Brassica napus cv Westar and hairy AtGL3^{+} *B. napus* plants were transformed using the method developed by Block and co-workers (1989) with modifications as developed by Delwin Epp, transformation technician at Agriculture and Agri-Food Canada, Saskatoon Research Centre (unpublished). *A. tumefaciens* strain GV3101 containing O-TTG1, K-TTG1 or the p79-103 empty vector control was grown overnight at 28^{0}C in 15 ml LB medium (see **Appendix 6.4**).

Seeds of *B. napus* cv Westar and AtGL3^{+} *B. napus* were rinsed with 70% ethanol and surface-sterilized in a 6% NaOCl containing 0.1% Tween 20 (Sigma, Missouri, USA) for 10 min. Seeds were rinsed three times in sterilized tap water, followed by a fourth rinse in sterilized

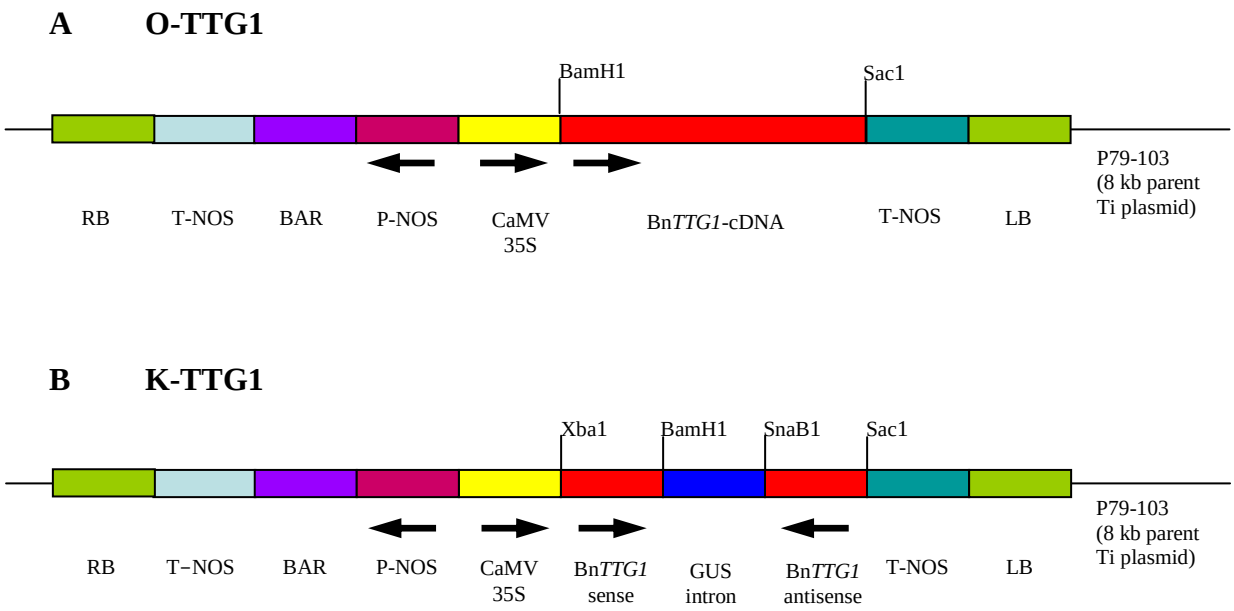


Fig. 2.1. *TTG1* binary vector TDNA used to transform *B. napus* cv Westar and AtGL3^{+} transgenic *B. napus*. (A) *BnTTG1* over-expression (O-TTG1) and (B) *BnTTG1* RNAi knock-down (K-TTG1). RB - Right border. LB – Left border. NOS - Nopaline synthase, BAR – phosphinothricin resistance selection gene

distilled water. Sterilized seeds were germinated on petri plates containing ½ MS medium (Sigma) with 2% sucrose and 0.8% agarose for 5 days in a tissue culture room with a 22/20^{0}C temperature regime and a 16/8 h light/dark photoperiod of 350 µE.m^{-2}.s^{-1}. Meristem and cotyledons were removed and hypocotyls were cut into 1 cm pieces. Hypocotyl segments were pre-cultivated on solidified co-cultivation medium (see **Appendix 6.4**) for 1 day. Pre-cultivated hypocotyl segments were co-cultivated with 1 X 10^{7} *Agrobacterium tumefaciens* cells/plate (12 ml) for two days. Hypocotyl segments were washed for 5 min with co-cultivation medium containing 450 mg/L timentin (Smith Kline Beecham, London, UK) to kill the *Agrobacterium* before transferring to solid callus induction medium (see **Appendix 6.4**; Hypo I). After two weeks, hypocotyl segments were transferred to solid callus regeneration / shoot induction medium (see **Appendix 6.4**; Hypo II) for 2 weeks. Calli with green shoots were transferred to a

plate containing solid shoot regeneration medium (see **Appendix 6.4**; Hypo III) for 2 weeks to develop young leaves. Plantlets with leaves were transferred to rooting medium (see **Appendix 6.4**; Hypo IV) in tall jars for 4-5 weeks to develop roots.

Once small plantlets had developed roots, phosphinothricin resistant plantlets were transferred into soiless mix (see **Appendix 6.4**) in 15 cm pots and grown in the greenhouse. The small plantlets were covered with clear plastic cups for the first few days after transplanting to help them acclimate to the greenhouse conditions. Prior to flowering, small samples of leaf tissue were harvested and frozen at -80^{0}C for confirmation of plant transformation.When plants started flowering, they were wrapped individually with perforated pollination bags (Cryovac Sealed Air, Canada) and the bags were gently shaken by hand once every two days to promote self-pollination. Mature seed pods were collected into paper bags and stored at room temperature.

2.2.3.2. Molecular characterization of transgenic plants

2.2.3.2.1. DNA extraction and PCR

Screening of putative phosphinothricin-resistant plantlets for positive transformants was carried out by PCR with several primer sets (35S-F, F/R BAR, F/R-GUS) listed in **Table 2.1** using leaves from plantlets which were firmly established and growing in the greenhouse. *BAR* gene specific primers were used for the screening of both over-expressed (O-TTG1) and knock-down (K-TTG1) T_0 transformants, whereas GUS intron specific primers were also used to screen putative knock-down transformants. Genomic DNA for PCR analysis was extracted from plants using the DNeasy® Plant Mini Kit. The final PCR mixture contained 1x PCR buffer, 1.5 mM $MgCl_2$, 200 mM dNTPs, 2 U Taq DNA polymerase (Invitrogen), 10 µM each of forward and reverse primers and 100 ng genomic DNA to a final volume of 20 µl with sterilized distilled water. The PCR conditions were: 94^{0}C/5 min, 40 x [94^{0}C/30 sec, 52^{0}C/30 sec, 72^{0}C/1 min], 72^{0}C/7 min and 4^{0}C/∞. PCR samples were separated on 0.8% agarose gels in 1x TAE buffer (see **Appendix 6.3**) and visualized by ethidium bromide staining.

2.2.3.2.2. RNA extraction and qRT-PCR

To measure expression of seven trichome-related genes and three anthocyanin-related genes, RNA was extracted from transgenic and non-transgenic plants using the RNeasy® Mini Kit, and 5 µg RNA samples were used for first strand cDNA synthesis. For the trichome gene

expression study, cotyledons were harvested from three-day-old seedlings, the first three leaves were harvested when the third leaf was still developing, and the 4^{th}-6^{th} leaves were harvested when the sixth leaf was still developing. First-strand cDNA synthesis was performed using SuperScript™ II RT (Invitrogen) with Oligo$(dT)_{12-18}$ primers. Final qRT-PCR mixtures contained 1x Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 10 µm each of gene-specific forward and reverse primers (**Table 2.2**), and 100 ng cDNA in a final volume of 20 µl with sterilized distilled water. The relative expression of *BnTTG1* in T_0 putative transformants was measured in leaves using a StepOne® thermal cycler (Applied Biosystems, California, USA). To measure the relative expression of primary trichome regulatory genes, flavonoid/anthocyanin genes and *LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE* (*ACT2*) as a qRT-PCR control gene, cDNA was assayed in a Bio-Rad CFX Manager thermal cycler (California, USA) using *ACT2* as the control gene from cotyledons, 1^{st}-3^{rd} leaves (combined) and 4^{th}-6^{th} leaves (combined) of non-transgenic and T2 homozygous transgenic *B. napus* lines grown in a growth chamber with a $22/18^0$C temperature regime and a 16/8 h light/dark or 24 h (continuous) photoperiod of 400µE.m^{-2}.s^{-1}. The PCR conditions were: 50^0C/2 min, 95^0C/2 min, 40 x [95^0C/15 sec, 60^0C/30 sec], 95^0C/10 sec, melting curve and 4^0C/∞.

2.2.3.2.3. Southern hybridization

For the preparation of a T-DNA specific hybridization probe, a 438 bp specific fragment of the *BAR* gene was amplified from the binary vector p79-103 as a template. The product was separated and analysed on a 0.8% agarose gel and the amplicon was isolated from the gel using the QIAquick® Gel Extraction Kit. Gel-purified PCR product (25 ng) was mixed with sterilized distilled water to 23 µl in a screw-cap 1.5 ml centrifuge tube, denatured in boiling water for 2-3 min and quickly cooled on ice for 2 min. The PCR product was radiolabeled using the Random Primers DNA Labeling System (Invitrogen) by adding 6 µl of a dNTP mix (G:A:T, 1:1:1), 15 µl of random primer/buffer mix, 1 µl of Klenow enzyme (3 U/ µl) and 5 µl ^{32}P-dCTP (specific activity > 1800 Ci/mmol) (PerkinElmer, Massachusetts, USA). The mixture was incubated at 37^0C for 30 min and centrifuged at 3000 rpm for 1 min through a ProbeQuant™ G-50 micro column (GE Healthcare, Little Chalfont, UK) to remove unincorporated radio-nucleotide.

Table 2.2. Primer sequences used in qRT-PCR analysis.

Name	Sequence (5' to 3')	Fragment size (bp)
QLGL1 QRGL1	CCACAAGCTACTTGGCAACA CCAGTGGTTTTGACAGCAGA	143
QLGL2 QRGL2	CGCTGGCCGGGAGAAAGAGC GGAGGTTTTTTCTGGATGAA	150
QLGL3 QRGL3	GGCTATGCAATGCTGGTACT CCTCTGCAATGTGTTCTGTG	150
QLTTG1 QRTTG1	CTCTGGGAGGTCAACGAA ATGCTGCACGTGCCTAAC	140
QLTRY QRTRY	TCGGTGATAGGTGGGATTTA GTGGACGATGAGGTTTGGTA	159
QLAtGL3 QRAtGL3	ACTTTCACCGGAAGATCTCG TTACTATCCGCCGTATGAGC	147
QLACT2 QRACT2	GATTGCTTAAAACGCTGCAT TTATTGGCACCACTGGAACT	154
QLEF1 QREF1	TTCACATCAACATCGTGGTC CACGCGTACTTGAAAGACCT	157
BnANS-Q-F BnANS-Q-R	AAAAAGCGGAATCAGCTCAA ACGGATGGTTTCGTCTTCTG	157
BnDFR-Q-F BnDFR-Q-R	CGTCTGCTGGAACGGTTAAT CGTAATCCCAAGCTGCTTTC	153
BnGST-Q-F BnGST-Q-R	CTCTAGAGCACCGAGCCATC CTTGAGCTCCTCGACCAAAG	146

Twenty micrograms of genomic DNA, extracted from young leaves of non-transgenic and transgenic plants using CTAB (see A**ppendix 6.3**) extraction (Murray and Thompson, 1980) were digested overnight at 37^0C with 40 U *Hind*III, selected to avoid cutting within the BAR probe sequence. The digested DNA was separated on a 1% agarose gel and transferred using paper towel blotting units overnight in 0.4 M NaOH to Amersham Hybond™-N^+ nylon membrane (GE Healthcare). The membrane was cross linked in a UVTrataLinker® 1800 (Stratagene, California, USA).

The membrane was prehybridized in 7 ml hybridization buffer (see **Appendix 6.3**) at 65^0C for at least 30 min in a rolling tube in a hybridization oven (Hybaid, Ontario, Canada). ^{32}P-labelled probe DNA was denatured by boiling for 3 min, quick cooled on ice and transferred to the bottom of the hybridization vial with new hybridization buffer. The membranes were hybridized with the probe overnight at 65^0C. Membranes were washed twice each with solution A (2x SSC, 0.1% SDS) for 15 min at 65^0C and solution B (0.2x SSC, 0.1 SDS) (see **Appendix 6.3**) for 15 min at 65^0C prior to autoradiography using Kodak scientific imaging film (BioMax XAR) at -80^0C for various time periods.

2.2.3.2.4. TDNA insert loci

Genome walking analysis was carried out to determine the TDNA insert loci using a GenomeWalker™ Universal Kit (Clontech) according to the manufacturer's instructions. Genomic DNA was extracted from young leaves of transgenic plants using the CTAB extraction method (see **Appendix 6.3**) and digested with *DraI* and *EcoRV* separately to develop libraries. Two libraries each for both K-5-8 and K-6-3 lines were developed. *BAR* gene-specific primers were used as gene-specific primer I and II. PCR products of each enzyme of each line were sequenced.

2.2.4. Microscopy of trichome bearing tissues

Small leaf disks (9 mm in diameter) of different tissues on the first six leaves of wild-type and transgenic *B. napus* lines were punched and directly mounted on aluminum stubs. Observations on trichome density and structure were made using a Philips 505 scanning electron microscope at an accelerating voltage of 30 kV in the microscopy facility of the Department of Biology, University of Saskatchewan. Images were photographed on FP-100B panchromatic

type Polaroid films (Fujifilm, Tokyo, Japan). Digital images were taken for counting trichome numbers. Number of trichomes on a 1 mm^2 area of the adaxial surface were counted and the numbers were adjusted to cm^2. For the petiole, leaf edge and abaxial surface on the mid vein, number of trichomes on a 1 mm length were counted and adjusted to cm. Trichome densities were measured three times (i.e. on 3 separate positions per leaf) on six leaves per plant on three independent plants per line.

To measure trichome length, trichomes were extracted from 1^{st}-4^{th} leaves of 3-week-old seedlings. To extract trichomes, leaf samples were vortexed in a 50 ml tube containing liquid nitrogen. Debris was manually removed and the remaining contents were filtered through a 40 µm nylon cell strainer (BD Falcon, California, USA) to retain trichomes. Trichomes were viewed under a ZEISS, Axio Vision epifluorescent microscope and lengths of unbroken, complete trichomes were measured from tip to base using an Axio Vision Documentation system (ZEISS, Jena, Germany).

2.2.5. Anthocyanin analysis

Anthocyanins were extracted from *B. napus* wild-type and transgenic seedlings grown in a growth chamber with a $22/18^0$C temperature regime and a 24 h (continuous) photoperiod of $400\mu E.m^{-2}.s^{-1}$ to enhance anthocyanin production. Above ground tissues of 10-day-old seedlings were ground under liquid nitrogen. Five hundred micro litres of extraction buffer (99% MeOH + 1% HCl) was added to 200 mg of ground tissue and samples were incubated for 24 h at 4^0C followed by centrifugation at 13000 g for 5 min to clarify the supernatant. Total anthocyanin was quantified against a standard curve for cyanidin chloride using an 1100 Series HPLC (Agilent Technologies, California, USA).

2.2.6. Seed mucilage analysis

Seed coat mucilage of *B. napus* was analyzed using a modified protocol (personal communication with C. Coutu - AAFC) for *Camelina sativa* seed mucilage screening. Ten seeds per line, were shaken at 210 rpm for 20 min in 1 ml 0.1% Nonidet-P40 detergent followed by washing with 1 ml of tap water. The hydration liquid was replaced with 250-300 µl of 0.7% (v/v) India ink in 0.1% Nonidet-P40 and left for one hour prior to photographing the seeds. To stain with Ruthenium Red, seeds were washed with 1 ml of tap water to remove India ink and replaced with 500 µl of 0.01% Ruthenium Red. Seeds were shaken at 210 rpm for 15 min, washed with

tap water followed by soaking in tap water for 5 min to allow unbound dye to diffuse out of the mucilage. The solution was replaced with 500 µl of fresh tap water and seeds were photographed.

2.2.7. Growth measurement conditions and design

For growth analysis, plants were cultivated in CoCo mix in 15 cm pots and grown in a greenhouse with a 22/18^{0}C temperature regime and a 16/8 h light/dark photoperiod (230 µE.m^{-2}.s^{-1}). A complete randomized design (CRD) was used with three replicates. Pots were covered with clear plastic flats until seeds germinated and small plantlets were acclimated to greenhouse conditions. Percent emergence was counted up to 10 days after seeding until all seeds were germinated. Height of each plant was measured using a ruler up to 6 weeks after seeding. When plants started flowering, they were wrapped individually with perforated pollination bags and gently shaken by hand once every two days to promote self-pollination. Number of days to pre-bolting (onset of flower buds), bolt initiation (appearance of peduncle) and flower opening (initial opening of flowers) from seeding were recorded for each plant. Mature seed pods were collected into paper bags and total seed weight per plant was measured. Hundred seed weight was measured using 100 randomly selected seeds per each plant.

2.2.8. Statistical analysis

Data were analyzed with either one way and two way ANOVA using a MIXED model in SAS 9.2 (SAS Institute, 2008). Assumptions of ANOVA were tested using a Normality test and Levene's test. Means were compared using a Tukey test in SAS 9.2 and treatments were declared significant at $P \leq 0.05$. Trends were declared at $P \leq 0.1$.

2.3. Results

2.3.1. Transformation of two *B. napus* backgrounds with *TTG1* constructs

Transformation of *B. napus* cv Westar hypocotyl segments with either over-expression and RNAi knock-down constructs each produced 20 individual phosphinothricin (PPT) tolerant putative transformants out of 1200 explants. However, transformation of AtGL3$^+$ *B. napus* (AtGL3$^+$) hypocotyl segments with over-expression and knock-down constructs resulted in the recovery of only nine and 10 phosphinothricin (PPT) tolerant putative transformants, respectively, from 1200 explants (**Table 2.3**).

Table 2.3. Transformation efficiency with *BnTTG1*; survival and transgene insertion pattern of confirmed transformants.

Background	**Binary construct**	**No. of explants**	**No. of putative T_0 PPT-tolerant plantlets**	**No. of confirmed T_0 transgenic events**	**No. of single T-DNA insertion plants**	**No. of T_0 plants that set seed**
B. napus Westar	O-TTG1	1200	20	12	5	10
	K-TTG1	1200	20	7	2	5
AtGL3$^+$ *B. napus*	O-TTG1	1200	9	7	2	1
	K-TTG1	1200	10	9	4	5

2.3.2. Molecular analysis of transgenic plants and development of advanced lines

2.3.2.1. PCR confirmation

Screening for positive transformants was carried out with several primer sets listed in **Table 2.1** using leaves of T_0 PPT-tolerant plantlets which were firmly established and growing in the greenhouse. While *BAR* gene-specific primers were used to confirm both over-expressed (O-TTG1) and knock-down (K-TTG1) T_0 transformants, GUS intron-specific primers were used as a second screen to confirm knock-down transformants (**Fig. 2.2**).

While screening of 20 *TTG1* over-expression T_0 transformants in the *B. napus* cv Westar background resulted in 12 positives (**Fig. 2.2A, Table 2.3**), knock-down transformation events in this background resulted in only seven confirmed transformants (**Fig. 2.2B**). This represented a 40-65% "leakage" (false positive) in the Westar background using PPT as the screen. Seven out of nine PPT-tolerant T_0 *B. napus* AtGL3$^+$ plants transformed with *35S:TTG1* were confirmed as positive, while TTG1-RNAi in the *B. napus* AtGL3$^+$ background resulted in nine transgenic plants (**Fig. 2.2C,D, Table 2.3**). In the AtGL3$^+$ background false positives were reduced to between 10-20%. Subsequent screening of T_1 and T_2 generations of transgenic plants (towards the production of homozygous lines) was conducted using the 35S-F sense primer and gene specific primers ORttg1 and LRttg1 for over-expressed lines and knocked-down lines, respectively (**Table 2.1**). Two independent homozygous lines, K-5-8 and K-6-3, for plants carrying the *BnTTG1* knock down in the *B. napus* AtGL3$^+$ background were developed. Only one independent homozygous line O-3-7 was developed for the over-expression construct in the same background due to the lack of plants that could set seed (**Table 2.3**).

2.3.2.2. Southern hybridization to determine inserts copy number

For the purpose of generating stable, single insertion, homozygous lines, T_0 and T_1 plants were screened by southern hybridization for the number of TDNA insertions. *BAR* gene-specific radio-labeled probes were used for the hybridization. Four of the nine confirmed K-TTG1 positive AtGL3$^+$ *B. napus* plants had a single TDNA insertion while two of nine O-TTG1 AtGL3$^+$ *B. napus* plants had a single insertion (**Fig. 2.3, Table 2.3**).

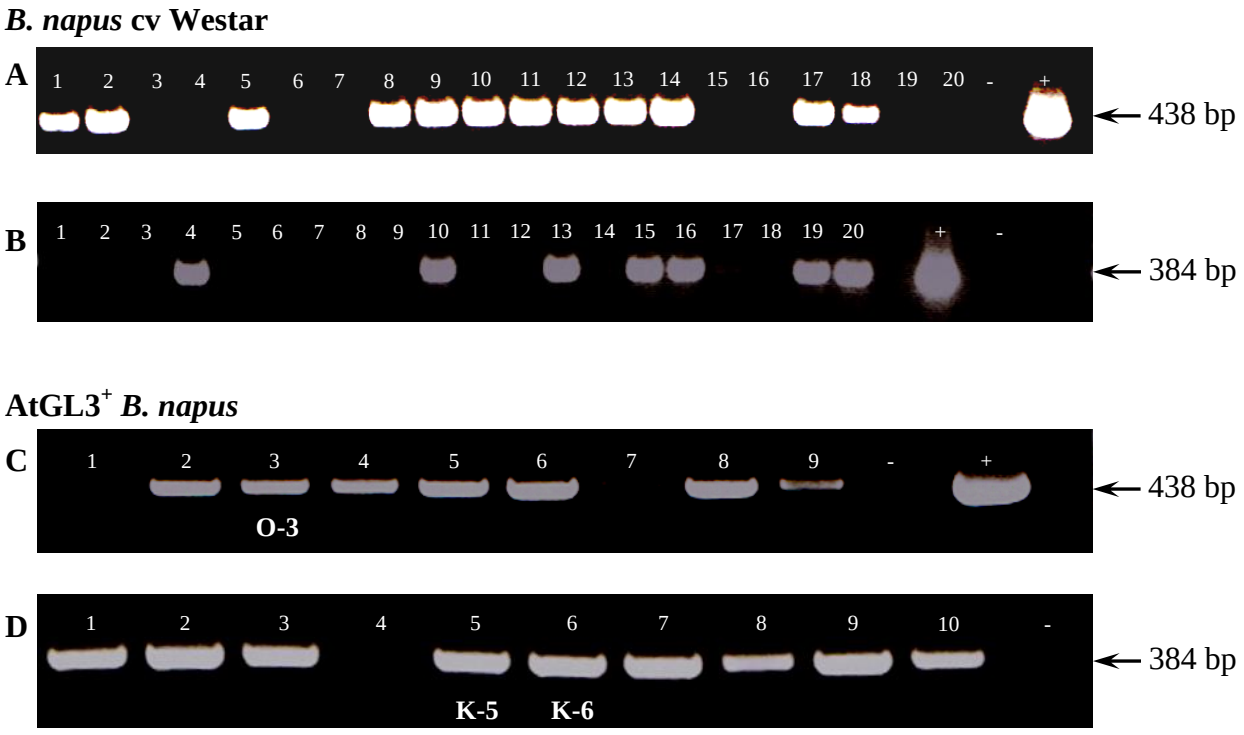


Fig. 2.2. PCR confirmation of O-TTG1 or K-TTG1 TDNA in putative phosphinothricin tolerant T_0 transformants in *B. napus* cv Westar background and AtGL3$^+$ *B. napus* (AtGL3$^+$) background. A) *BAR* gene specific primers for O-TTG1 in Westar. Lane 1-20, 20 putative transgenic Westar plants. B) GUS intron specific primers for K-TTG1 in Westar. Lane 1-20, amplification products from 20 putative transgenic Westar plants. Lane (-), amplification product from a Westar untransformed plant (A, B); Lane (+), amplification product from the binary vector containing (A) *BnTTG1* cDNA, (B) *BnTTG1*-RNAi (positive control). C) *BAR* gene specific primers detecting O-TTG1 in the AtGL3$^+$ background. Lane 1-9, amplification products from nine putative transgenic AtGL3$^+$ plants. D) GUS intron-specific primers detecting K-TTG1 in the AtGL3$^+$ background. Lane 1-10, amplification products from ten putative *BnTTG1* knock-down transgenic AtGL3$^+$ plants. Lane (-), amplification product from a AtGL3$^+$ transformed control plant not carrying *BnTTG1* (C, D); Lane (+), amplification product from the binary vector containing *BnTTG1* cDNA (positive control). Transgenic lines O-3, K-5 and K-6 were used to develop homozygous lines.

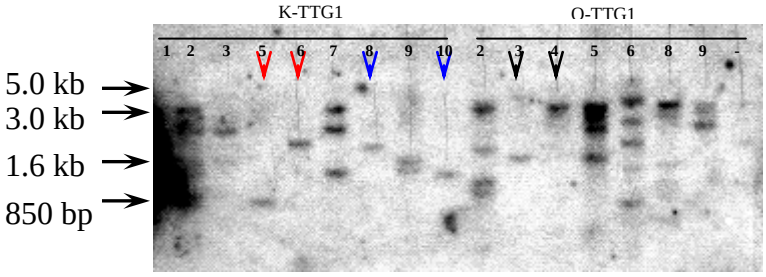


Fig. 2.3. Southern analysis to detect copy number of K-TTG1 and O-TTG1 TDNA in the AtGL3^{+} *B. napus* (AtGL3^{+}) background. Genomic DNA (20 µg) extracted from T_0 plants of K-TTG1 and O-TTG1 in the AtGL3^{+} background was digested with *Hind*III and probed with a ^{32}P-labeled *BAR* gene-specific probe. From the left; Lanes 1-10, DNA from nine individual K-TTG1 T_0 plants; Lanes 2-9, DNA from seven individual O-TTG1 T_0 plants; Lane (-) DNA from a non-transformed AtGL3^{+} control plant. Red arrows, single insertion K-lines with an enhanced trichome phenotype (plants 5 and 6). Blue arrows, single insertion K-lines with AtGL3^{+}-like trichome phenotype (plants 8 and 10). Black arrows, two single insertion O-lines lacking trichomes.

Hybridization of the *BAR* gene-specific radio-labeled probe with six seedlings each from the three positive K-TTG1 T_1 transformants K-4, K-10, K-16 in *B. napus* cv Westar confirmed two parent lines, K-4 and K-16 with single TDNA insertions (**Fig. 2.4**).

Five T_0 independent single insertion plants were identified for the over expression of *BnTTG1* in *B. napus* cv Westar background (**Fig. 2.5**).

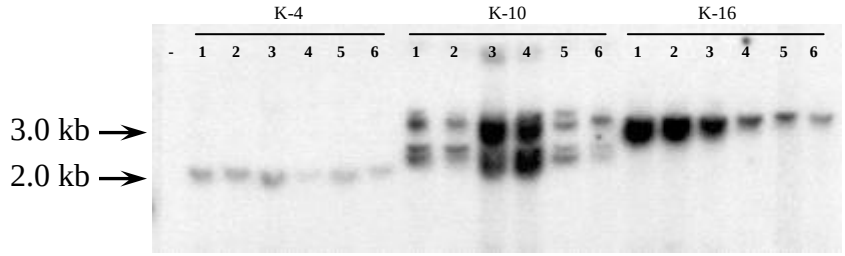


Fig. 2.4. Southern analysis to detect copy number of the K-TTG1 transgene in *B. napus* cv Westar. Genomic DNA (20 µg) extracted from T_1 K-TTG1 plants K-4, K-10, K-16 in Westar was digested with *Hind*III and hybridized to a ^{32}P-labeled *BAR* gene-specific probe. From the left; DNA from Westar non-transgenic control plant (-), DNA from six seedlings each of the three T_1 independent transgenic events K-4, K-10 and K-16.

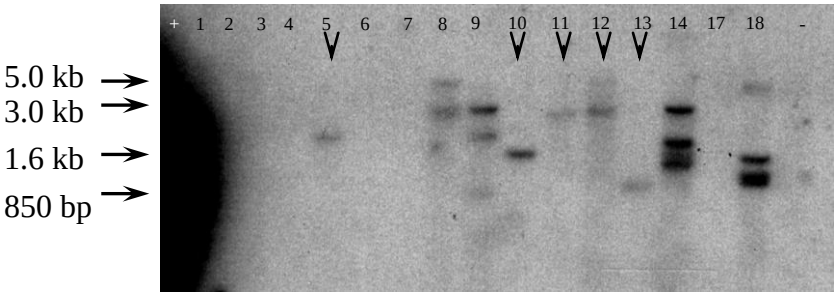


Fig. 2.5. Southern analysis to detect copy number of the O-TTG1 transgene in *B. napus* cv Westar. Genomic DNA (20 µg) extracted from T_0 plants of O-TTG1 in Westar background was digested with *Hind*III and hybridized to a ^{32}P-labeled *BAR* gene-specific probe. From the left; Positive control plasmid DNA (+); Lane 1-18, DNA from 16 individual O-TTG1 T_0 plants; DNA from Westar non-transgenic control plant (-). Black arrows, five single insertion O-lines.

2.3.2.3. Relative expression of *BnTTG1* in T_0 putative transformants

Relative expression of *BnTTG1* in putative transformants was measured in leaf tissue of greenhouse-grown T_0 plantlets using quantitative Real Time PCR (qRT-PCR). Six out of seven positive transformants of K-TTG1 in the *B. napus* cv Westar back ground showed reduced levels of *BnTTG1* relative to that of untransformed control plants (**Fig. 2.6**). While plant line 13 showed the lowest relative expression (10-fold reduction), line 20 had an identical expression level to that of the Westar control. The two single insertion lines, K-4 and K-16, showed 5-fold and 0.75-fold lower relative expression levels compared to that of the multiple insertion K-10 line.

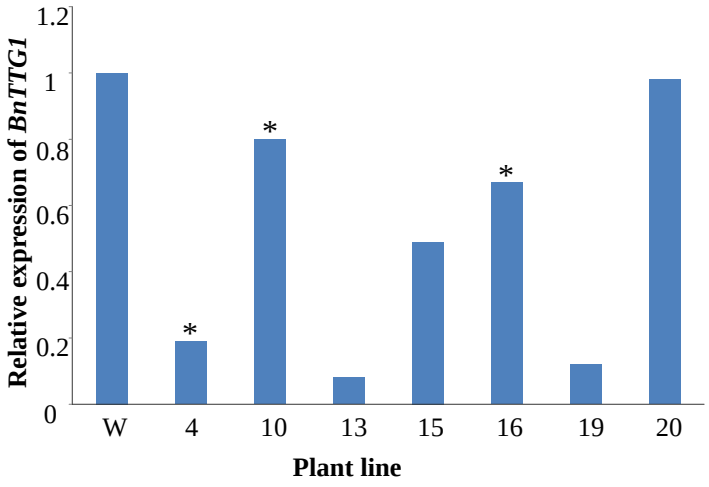


Fig. 2.6. Relative expression of *BnTTG1* in seedling tissues of seven individual T_0 K-TTG1$^+$ *B. napus* cv Westar plants using qRT-PCR. Expression is relative to *BnTTG1* expression in Westar seedling tissues (set at 1), which was normalized to the expression of the *B. napus* gene *ACT2*. Plant lines 4 and 16 each have single TDNA insertions and plant 10 has multiple insertions (*) (see **Fig. 2.4**). All plants show Westar-like trichome phenotype. Three technical replicates were carried out for each plant.

Relative expression of *BnTTG1* was also measured in 11 positive T_0 transformants of O-TTG1 in *B. napus* cv Westar (**Fig. 2.7**). A range of relative expression levels was observed for the 11 plants, with the highest expression (~ 70-fold increase) observed in plant line 12. Plant lines 2, 9 and 11 showed an expression level similar to that of untransformed Westar.

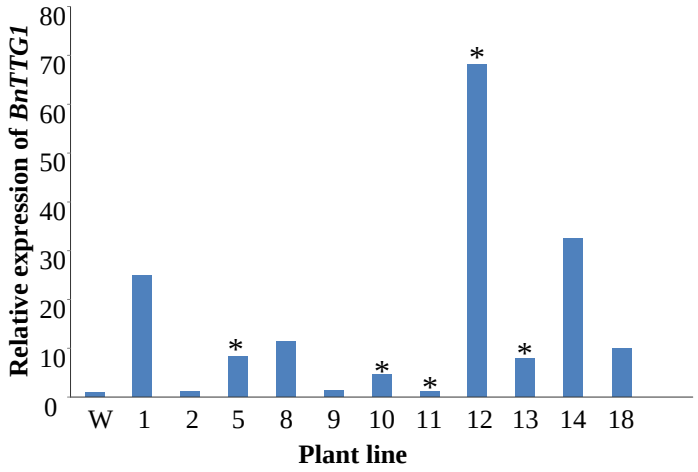


Fig. 2.7. Relative expression of *BnTTG1* in seedling tissues of 11 individual T_0 O-TTG1 Westar plants using qRT-PCR. Expression is relative to *BnTTG1* expression in Westar seedling tissues (set at 1), which was normalized to the expression of the *B. napus* gene *ACT2*. Lines 5, 10, 11, 12 and 13 have single TDNA insertions (*) (see **Fig. 2.5**) and all other lines have multiple insertions. All plants show Westar-like trichome phenotype. Three technical replicates were carried out for each analysis.

The four single insertion lines of K-TTG1 in the AtGL3$^+$ *B. napus* background were analyzed for *BnTTG1* expression (**Fig. 2.8**). The highest reduction (~ 20-fold reduction) was observed in line 5, followed by line 6 with an ~ 10-fold reduction compared to the control AtGL3$^+$ plant. However, the reduced levels observed in lines 8 and 10 were less extreme than those of lines 5 and 6. Both lines 5 and 6 showed a similar, more extreme trichome phenotype compared to AtGL3$^+$ plants whereas, trichome phenotypes of lines 8 and 10 were similar to that of AtGL3$^+$ plants. Therefore, lines 5 (K-5-8) and 6 (K-6-3) were developed into stable homozygous single insertion, transgenic lines.

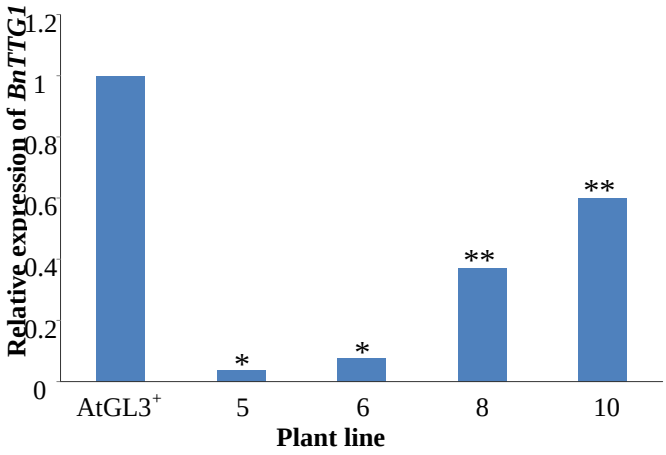


Fig. 2.8. Relative expression of *BnTTG1* in seedling tissues of four individual T_0 K-TTG1 AtGL3$^+$ *B. napus* plants (5, 6, 8 and 10) using qRT-PCR. Expression is relative to *BnTTG1* expression in AtGL3$^+$ seedling tissues (set at 1), which was normalized to the expression of the *B. napus* gene *ACT2*. Lines 5 and 6 have a single TDNA insertion (*) and an enhanced trichome phenotype (see **Fig. 2.3**) and thus were used to develop homozygous lines. Plants 8 and 10 have a single TDNA insertion (**) with a AtGL3$^+$ trichome phenotype (see **Fig. 2.3**). Three technical replicates were carried out for each line.

2.3.3. Determination of TDNA insert loci in K-5-8 and K-6-3 and their effect on trichome patterning

To determine whether the new extreme trichome phenotype in the K-5-8 and K-6-3 super-hairy lines arose as a result of gene disruption by the T-DNA insert, genome-walking was performed to identify the site of insertion. In K-5-8, the TDNA is located ~ 2 kb downstream of the *B. napus* ortholog of the Arabidopsis *BOI-RELATED GENE2* (*BRG2*) (AT1G79110, E value: 4e-59) gene, the protein product of which is involved in resistance to *Botrytis cinerea* (**Fig. 2.9A**). There was no orthologous region in Arabidopsis within ~ 2 kb downstream of the TDNA insert. The TDNA insertion in line K-6-3 is inside the *B. napus* ortholog of the Arabidopsis *SKU5 SIMILAR 2* (*SKS2*) (AT5G51480, E value: 2e-68) gene, the protein product of which functions in oxidoreductase activity, copper ion binding and is located in the plasma membrane (**Fig. 2.9B**).

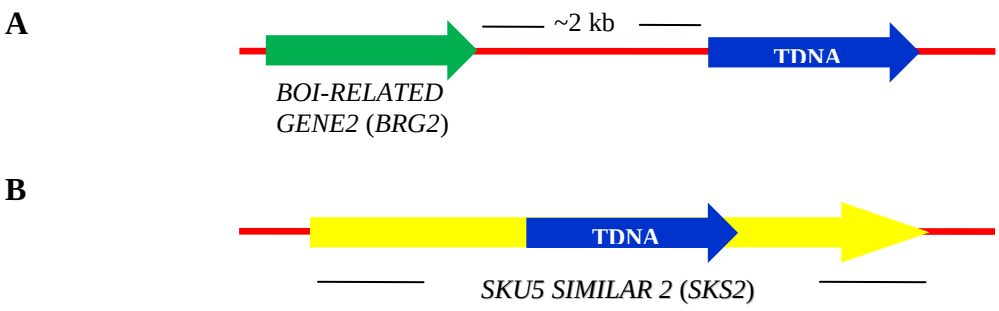


Fig. 2.9. TDNA insertion locations in the two most hairy lines A) K-5-8 and B) K-6-3 based on genome walking analysis.

To confirm that these two genes are not involved in trichome development and do not contribute to the hairy phenotype, SALK (Arabidopsis) knock-out lines (SALK_127570, SALK_074628, SALK_070108C, SALK_074627, SALK_127112C, SALK_070255 and SALK_036510) for these genes were obtained and trichome phenotypes of each were observed under the epifluorescent microscope. None of the SALK Arabidopsis lines showed any difference in either trichome density, distribution or morphology (**Fig. 2.10**) confirming that the increased trichome phenotypes observed in the K-5-8 and K-6-3 lines are not due to a gene disruption at the TDNA insertion site.

2.3.4. Morphological and agronomical phenotypes

Over-expression or knock-down of *TTG1* in a *B. napus* cv Westar background did not show any phenotypic difference with respect to trichome density or growth. Plants were healthy and growth and reproduction were similar to Westar non-transgenic control plants under greenhouse conditions. In contrast, over-expression (O-lines) or knock-down (K-lines) of *TTG1* in the AtGL3^{+} *B. napus* background showed substantial phenotypic changes (**Section 2.3.4.1**).

O-lines: Of the seven positive *TTG1* T_0 over-expression events in the AtGL3^{+} *B. napus* background, five showed slow growth and did not flower, eventually dying. However, two T_0 O-lines O-3 and O-9 did flower after transfer to soil. Only O-3 contained a single transgene insertion (**Fig. 2.2**). Both O-3 and O-9 lacked trichomes and were taller than the AtGL3^{+}

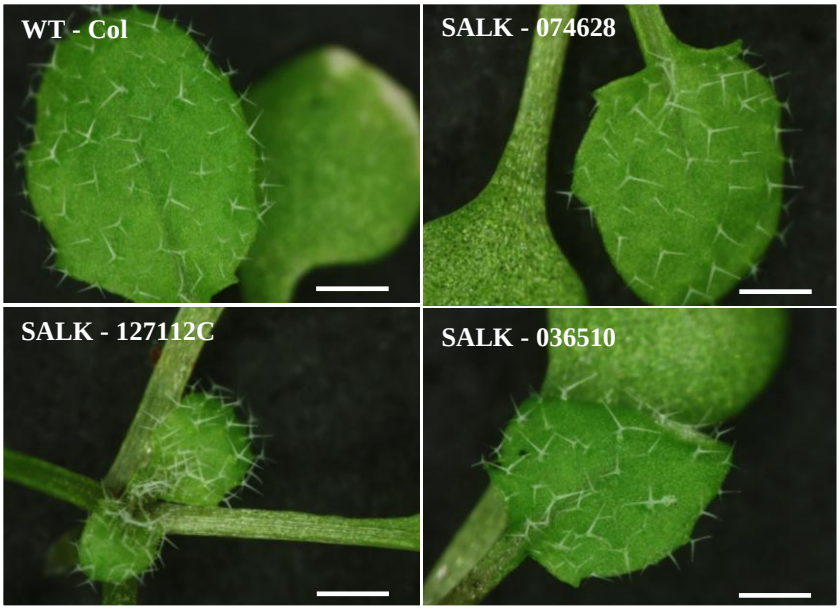


Fig. 2.10. Fourth rosette leaf trichome phenotypes of two-week-old *Arabidopsis thaliana* SALK lines 074628 (*BRG2*), 127112C (*BRG2*) and 036510 (*SKS2*). WT-Col, Columbia ecotype. Size bar represents 1 mm.

B. napus controls. Only O-3 produced a few pods with less than 5 seeds. The germination rate of these T_1 seeds was very low, but a lack of trichomes and a taller phenotype (compared to control plants) was visible in these T_1 plants. The germination rate of subsequent T_2 seeds (O-3-7) was also low and consistent with T_1 seeds. Out of 10 T_2 seeds, only three germinated and only one of the resulting plants flowered. Leaves of this O-3-7 plant were dark green in colour with no trichomes. Growth of T_2 O-3-7 was slow compared to AtGL3$^+$ control plants, but faster than that of T_1 O-3-7 plants. The T_2 O-3-7 plant was taller, flowered later and produced healthy but fewer pods compared to that of AtGL3$^+$ control plants (**Fig. 2.11**). PCR analysis of T_3 seedlings confirmed that this di-genic line is homozygous and that both transgenes (AtGL3$^+$ and *BnTTG1*) are present.

K-lines: Introduction of the *TTG1*-RNAi construct into the AtGL3$^+$ *B. napus* background resulted in nine independent positive knockdown events. Four of these T_0 plants contained a

single transgene insertion (**Fig. 2.3**) and presented two different trichome phenotypes. PCR analyses were conducted on T_1 and T_2 plants, and homozygous K-lines were developed for two independent transgenic events (K-5-8 and K-6-3) representing increased trichome phenotypes. Line K-5-8 with the most extreme trichome phenotype was used for further analysis. In the greenhouse, K-5-8 grew more vigorously and faster resulting in more robust plants than Westar and AtGL3$^+$ transgenic *B. napus* control plants (**Fig. 2.11**). AtGL3$^+$ *B. napus* control plants always showed the weakest growth compared to K-5-8 and Westar.

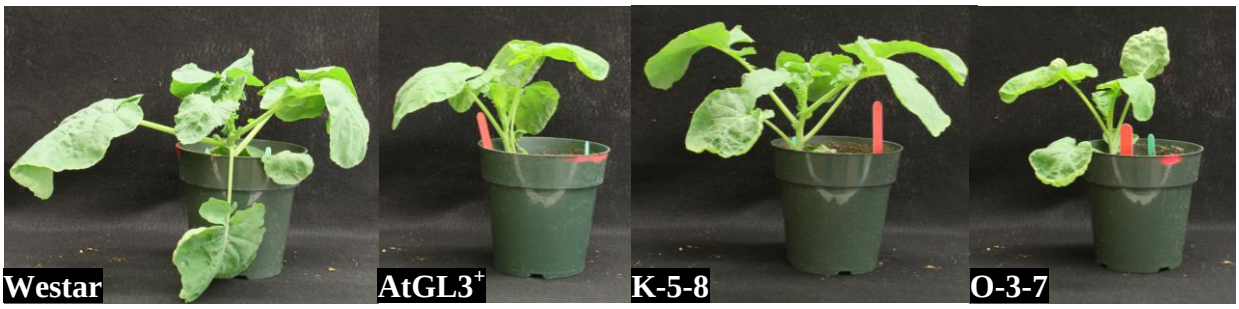


Fig. 2.11. Representative plants of four-week-old lines of homozygous *B. napus* cv Westar, AtGL3$^+$, K-5-8 and O-3-7 under greenhouse condition.

Differences in trichome phenotypes were visible between the three lines (glabrous Westar) even to the naked eye. As with Westar, cotyledons of the two transgenic lines, AtGL3$^+$ *B. napus* and K-5-8, developed in the Westar background, lacked trichomes (**Fig. 2.12A, D**). The first three true leaves and petioles of both transgenic lines showed the same increased trichome phenotype compared to Westar leaves and were indistinguishable from each other either to the naked eye or under a dissecting microscope (**Fig. 2.12B, E**). However, trichome densities and distribution patterns were distinctive between the two lines after the third leaf stage. While the trichome densities on leaves of AtGL3$^+$ *B. napus* control plants decreased at the fourth leaf stage (**Fig. 2.12C, F**), K-5-8 plants continued to display trichomes up to the 11th or 12th leaf stage. Differences in the height of trichomes were also observed between the two lines (discussed later in **Fig. 2.23**).

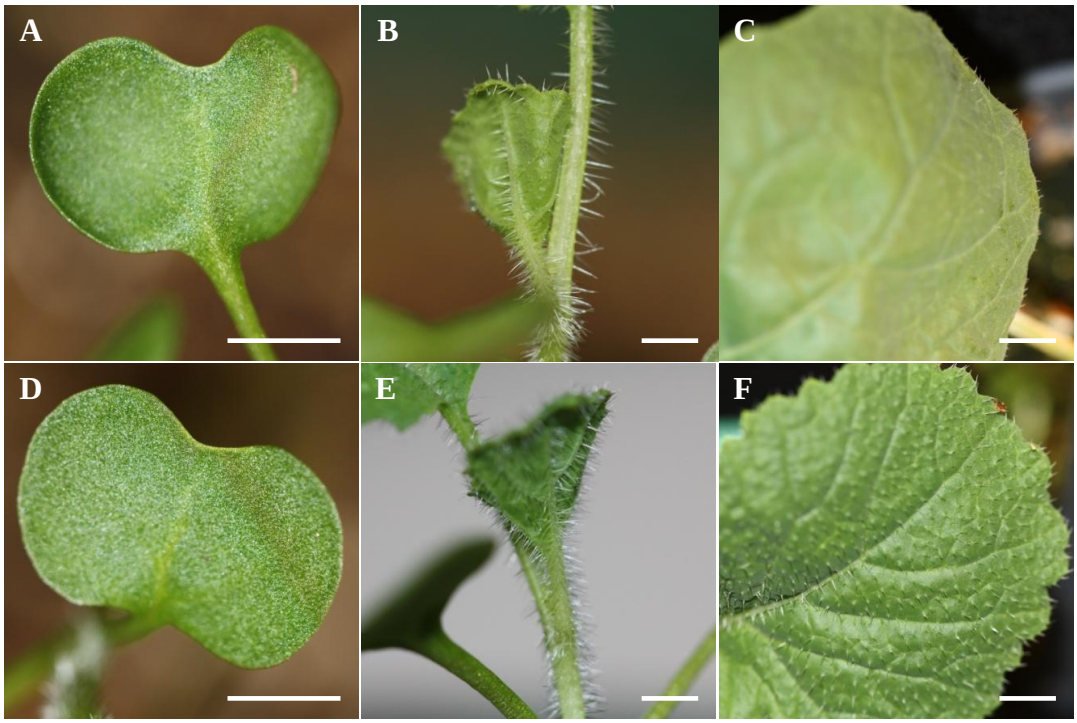


Fig. 2.12. Trichomes on the cotyledons, second leaf (10 DAG) and fourth leaf (3 WAG) of homozygous *B. napus* hairy seedlings under greenhouse conditions. A, B, C) AtGL3^{+} *B. napus*. D, E, F) K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A and D show glabrous cotyledons; B and E show trichomes on an emerging second leaf of both plant lines; C and F show lack of trichomes (AtGL3^{+}) and trichomes (K-5-8) on the fourth leaf. DAG – days after germination, WAG – weeks after germination. Size bar represents 1 cm.

Large numbers of trichomes were also observed on the stems of K-5-8 plants above the first node, a phenotype not present in AtGL3^{+}control plants (**Fig. 2.13**). The increased leaf and stem trichome phenotype that was more easily observed at the nodes of the K-5-8 line, was observed through five generations of plants, suggesting that the phenotype is stable. The occasional trichome was also present on the peduncle of K-5-8 plants, while this tissue in control plants was always glabrous (data not shown).

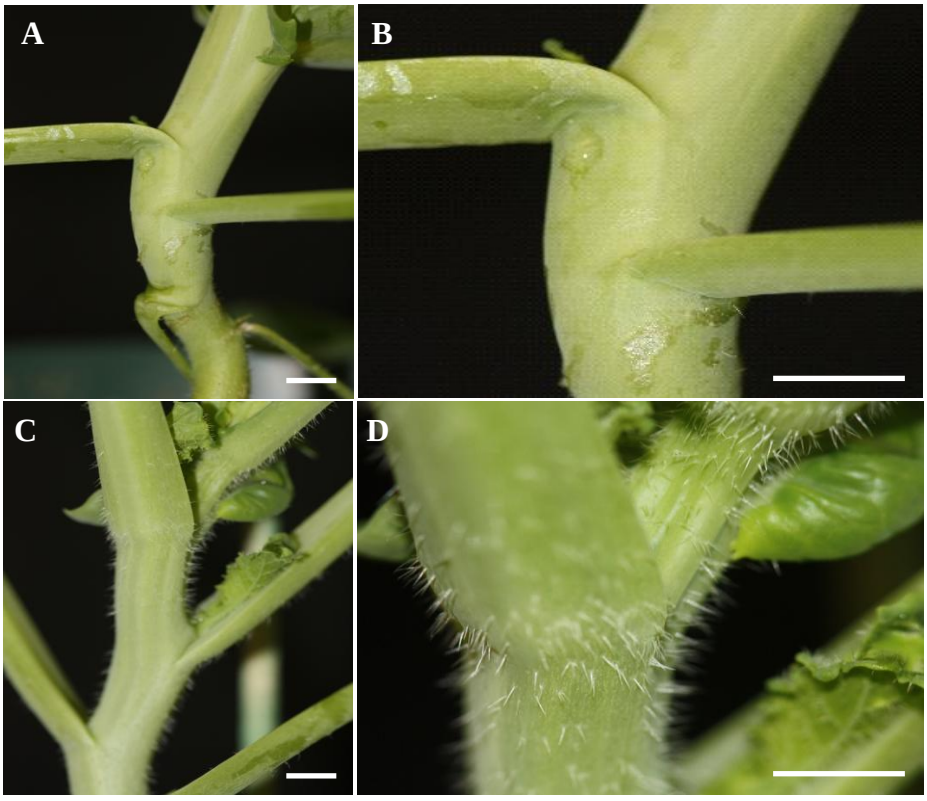


Fig. 2.13. Stem trichomes on hairy *B. napus* T_3 plants at eight WAG under greenhouse conditions. A, B) AtGL3^{+} *B. napus*. C, D) K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). B and D are 2.5-fold magnifications of A and C. WAG – weeks after germination. Size bar represents 1 cm.

2.3.4.1. Trichome density and distribution on the K-5-8 line

To study the densities and the distribution pattern of trichomes, the first six leaves of AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8, were observed under the scanning electron microscope (SEM). Since there was a difference in the trichome densities at different positions on the same leaf under the dissecting microscope, leaf edge, leaf adaxial surface, leaf abaxial surface along the mid vein, and petiole were selected for study (**Fig. 2.14-21**).

Both lines showed a similar high density trichome phenotype on the edges of the first to third leaves (**Fig. 2.14A, D**). However, while K-5-8 continued to produce high trichome densities even on the sixth leaf, trichome numbers declined by the fourth leaf of AtGL3^{+} plants, with no trichomes present on the sixth leaf (**Fig. 2.14B, C, E, F**).

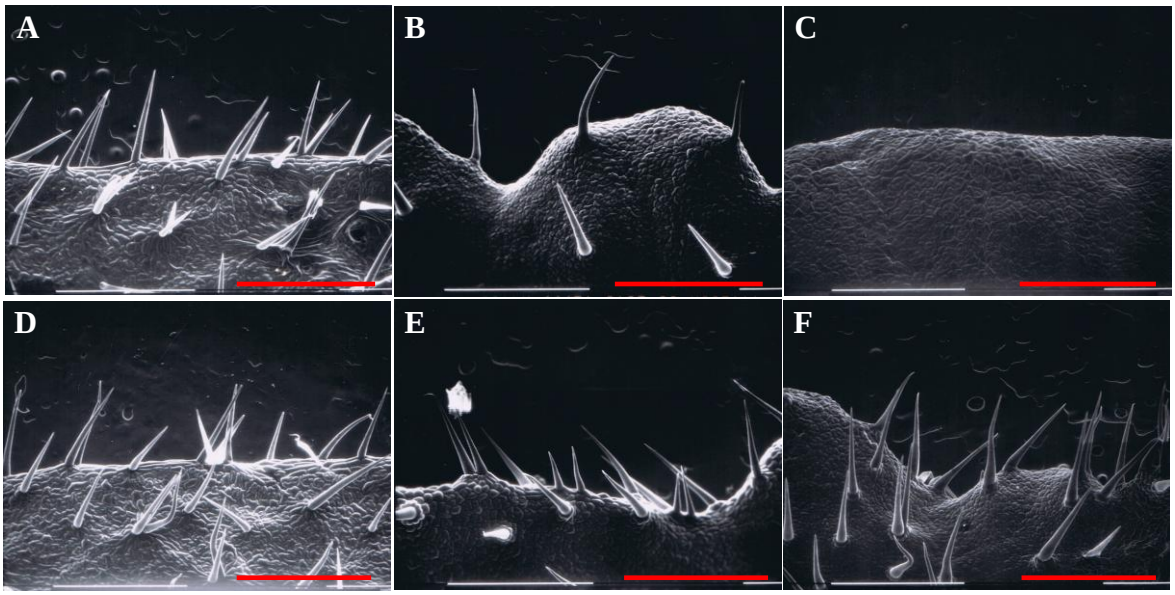


Fig. 2.14. Scanning electron micrographs showing representative distribution of trichomes along the edges of first, fourth and sixth leaves of *B. napus* hairy seedlings. A, B, C) AtGL3^{+} *B. napus*. D, E, F) T_3 K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A, D) first leaf 17 DAG. B, E) fourth leaf 24 DAG. C, F) sixth leaf 49 DAG. Size bar represents 1 mm. DAG – days after germination.

Mean trichome densities provided more detail to these visual phenotypes. The number of trichomes on the edges of the first two leaves of K-5-8 were significantly higher ($P \leq 0.05$) than that of the AtGL3$^+$ plants, while third leaves of both plant types were similar. The higher trichome density appeared again at the fourth leaf of K-5-8 and continued. The edges of the fourth leaf of K-5-8 had more than double the number of trichomes compared to that of the fourth leaf of AtGL3$^+$ and this increased further on the fifth and sixth leaves of K-5-8 (**Fig. 2.15**). While the sixth leaf of AtGL3$^+$ had a mean of two trichomes per cm on the edges, K-5-8 leaves had over 30 trichomes per cm, more than a 15-fold increase.

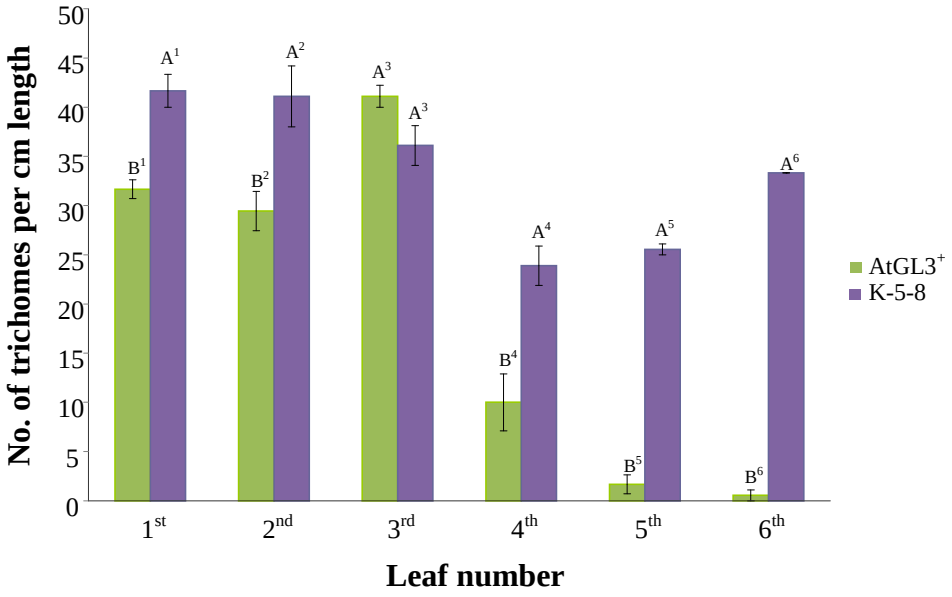


Fig. 2.15. Mean trichome densities along 1 cm edges randomly selected across the first-to-sixth leaves of AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*). Trichome densities were measured on first and second leaves from seedlings two WAG and third – sixth leaves from seedlings three WAG. A Tukey test was carried out between each of the plant lines for each leaf. Means of three plants per line (± standard error) with same letters do not differ significantly ($P \leq 0.05$). Trichome densities were measured three times (i.e. on three separate positions per leaf) on three independent plants per line. WAG – weeks after germination. Numbers in superscript indicate that there is no cross comparison between tissues and the comparison is only between lines.

The adaxial surface of leaves of both AtGL3^{+} and K-5-8 showed high numbers of trichomes (**Fig. 2.16**). However, K-5-8 plants produced significantly more trichomes on the adaxial surface of 1st-6th leaves than AtGL3^{+} plants (**Fig. 2.17**).

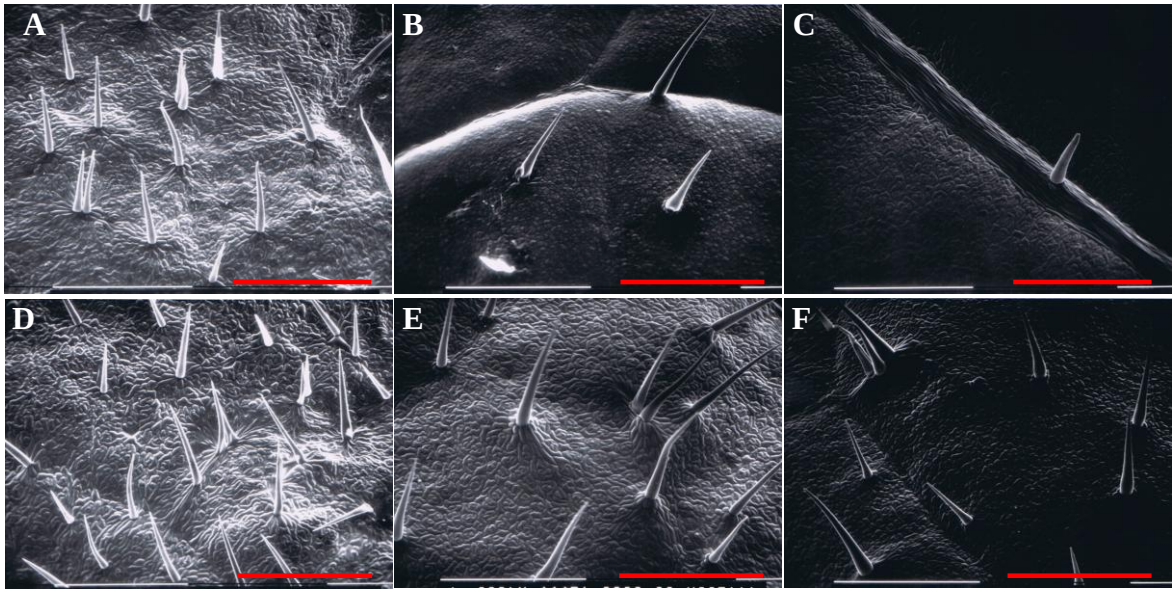


Fig. 2.16. Scanning electron micrographs showing representative distribution of trichomes on the adaxial surface of first, fourth and sixth leaves of *B. napus* hairy seedlings. A, B, C) AtGL3^{+} *B. napus*. D, E, F) T_3 K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A, D) first leaf 17 DAG. B, E) fourth leaf 24 DAG. C, F) sixth leaf 49 DAG. Size bar represents 1 mm. DAG – days after germination.

When trichome densities on adaxial surface were compared between fully developed first and second leaves of both lines, first leaves always had more trichomes than second leaves (**Fig. 2.17**). On average, the first leaves of K-5-8 had 75 more trichomes per cm^2 than AtGL3^{+}, with this difference increasing to 100 trichomes per cm^2 at the second leaf stage, and continuing to increase at the third leaf stage due to a decrease in number of trichomes on AtGL3^{+} leaves after the second leaf. While the sixth leaf of K-5-8 produced more than 250 trichomes per cm^2, AtGL3^{+} plants produced fewer than 10 trichomes per cm^2 and these densities further decreased

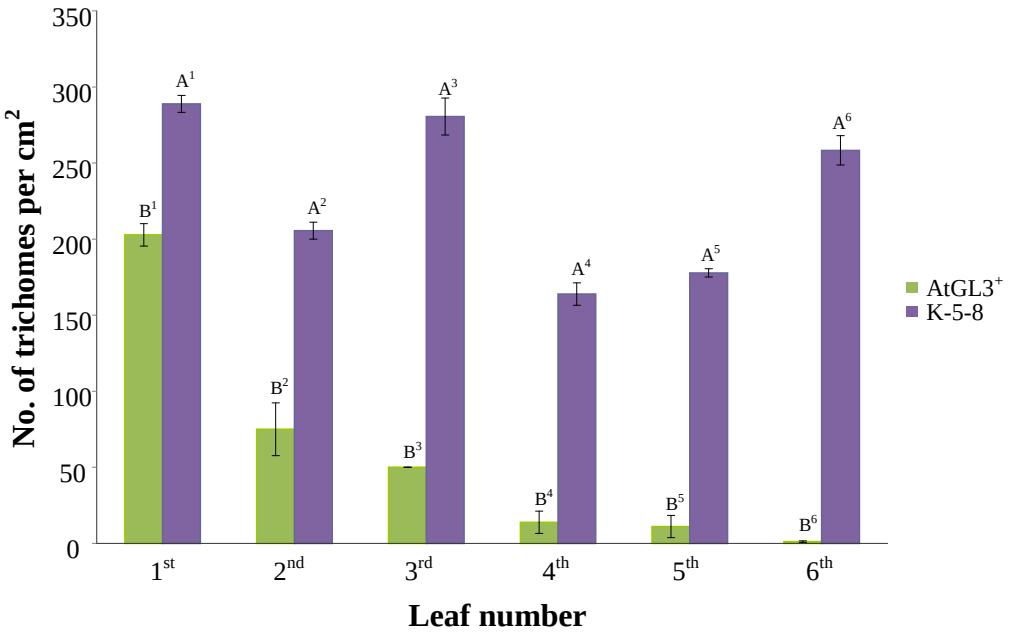


Fig. 2.17. Mean trichome densities on 1 cm^2 adaxial surface areas randomly selected across first-to-sixth true leaves of AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*). Trichome densities were measured on first and second leaves from seedlings two WAG and third – sixth leaves from seedlings three WAG. A Tukey test was carried out between the two plant lines for each leaf. Means of three plants per line (± standard error) with the same letters do not differ significantly ($P \leq 0.05$). Trichome densities were measured three times (i.e. on three separate positions per leaf) on three independent plants per line. WAG – weeks after germination. Numbers in superscript indicate that there is no cross comparison between tissues and the comparison is only between lines.

with the expansion of the AtGL3$^+$ leaf making it virtually glabrous and similar to *B. napus* cv Westar wild type plants at maturity. In contrast, the first leaf up to the sixth leaf of K-5-8 continuously produced significantly ($P \leq 0.05$) higher numbers of trichomes compared to AtGL3$^+$ (**Fig. 2.17**).

AtGL3$^+$ plants produced large numbers of trichomes on the abaxial surface mid vein of first to third leaves, and this high trichome density subsequently declined from the fourth to the

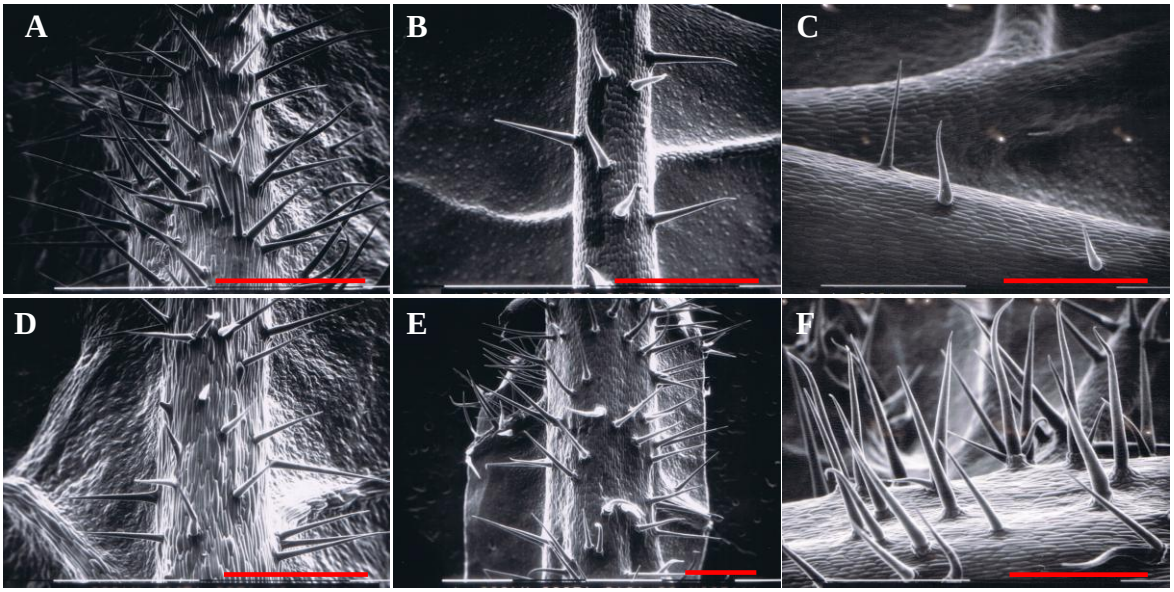


Fig. 2.18. Scanning electron micrographs showing representative distribution of trichomes on the abaxial surface of the mid vein of first, fourth and sixth leaves of *B. napus* hairy seedlings. A, B, C) AtGL3^{+} *B. napus*. D, E, F) T_3 K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A, D) first leaf 17 DAG. B, E) fourth leaf 24 DAG. C,F) sixth leaf 49 DAG. Size bar represents 1 mm. DAG – days after germination.

sixth leaf (**Fig. 2.18**) in a similar fashion to that at leaf edges (**Fig. 2.15**). However, the K-5-8 line continuously produced higher trichome densities even on the later developed leaves. Unlike the edge and adaxial surface pattern, the number of trichomes on the abaxial surface of ATGL3^{+} plants was substantially higher ($P \leq 0.05$) than that for K-5-8 on first and third leaves (**Fig. 2.19**). However, this difference was not significant at the second leaf stage. Trichome number was three-fold higher on the fourth leaf of K-5-8 compared to its AtGL3^{+} counterpart and these differences increased substantially on the fifth and sixth leaves ($P \leq 0.05$) (**Fig. 2.19**).

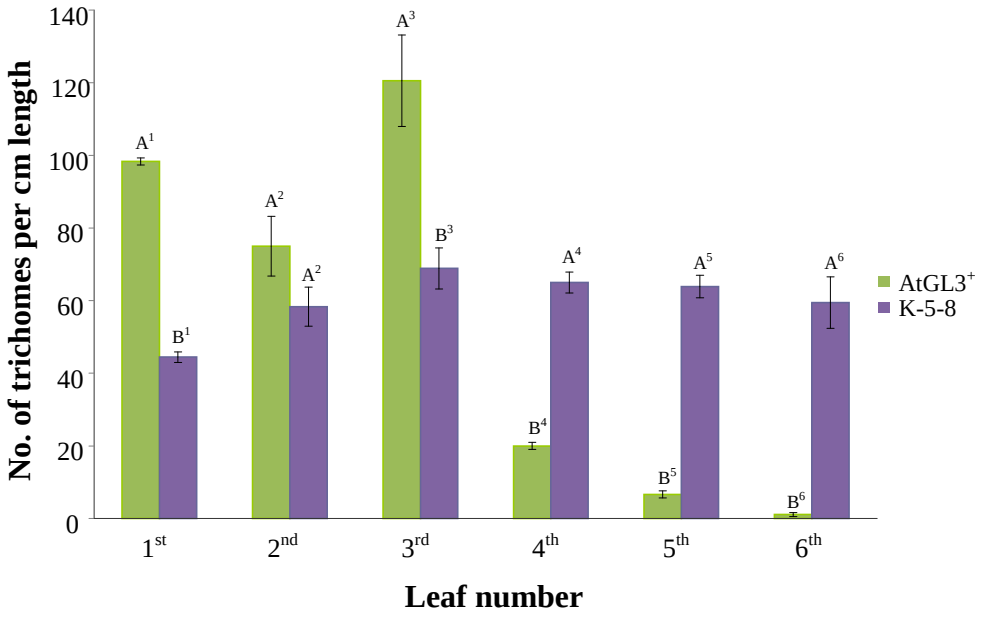


Fig. 2.19. Mean trichome densities along a 1 cm length of the abaxial surface of the mid vein on randomly selected first-to-sixth leaves of AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). Trichome densities were measured on first and second leaves from seedlings two WAG and third – sixth leaves from seedlings three WAG. A Tukey test was carried out between each of the plant lines at each leaf. Means of three plants per line (± standard error) with same letters do not differ significantly ($P \leq 0.05$). Trichome densities were measured three times (i.e. on three separate positions per leaf) on three independent plants per line. WAG – weeks after germination. Numbers in superscript indicate that there is no cross comparison between tissues and the comparison is only between lines.

A high number of trichomes were visible on the petioles of the AtGL3^{+} and K-5-8 plants, but densities declined on the fourth leaf petiole of AtGL3^{+} plants (**Fig. 2.20**). Trichome densities further decreased on AtGL3^{+} leaf petioles at the fifth leaf stage, such that the petiole of the sixth leaf was completely glabrous (**Fig. 2.20**).

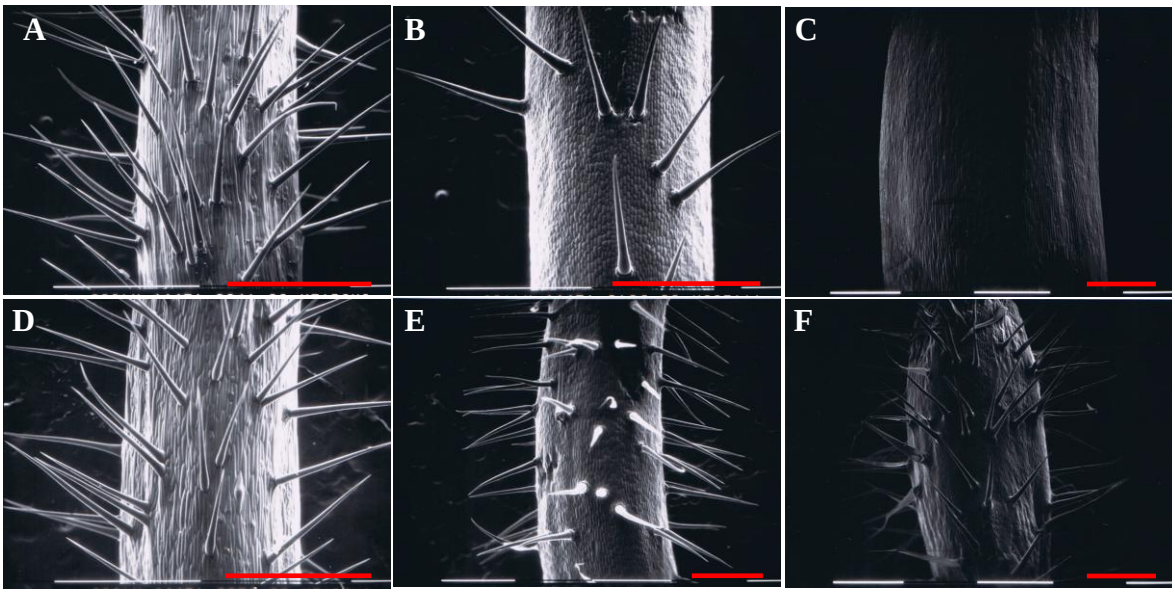


Fig. 2.20. Scanning electron micrographs showing representative distribution of trichomes on petioles of first, fourth and sixth leaves of *B. napus* hairy seedlings. A, B, C) AtGL3^{+} *B. napus*. D, E, F) T_3 K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A, D) first leaf 17 DAG. B, E) fourth leaf 24 DAG. C,F) sixth leaf 49 DAG. Size bar represents 1 mm. DAG – days after germination.

Similar to trichomes on the abaxial surface, petioles of the first two leaves of AtGL3^{+} plants had more trichomes than K-5-8 (**Fig. 2.21**). Differences were substantially higher between the two plant types at the first and second leaf stage ($P \leq 0.05$) where trichomes on AtGL3^{+} exceeded 200 per cm petiole length (**Fig. 2.21**). However, no difference in petiole trichome numbers for the third leaf was observed between the two lines, whereas the petiole of the fourth leaf of K-5-8 had two-fold greater trichomes than AtGL3^{+}. This pattern of decreasing trichome density on the petioles of AtGL3^{+} plants continued up to the sixth leaf stage, while trichome densities for petioles of these older K-5-8 leaves were slightly higher than that for the first leaf (**Fig. 2.20**).

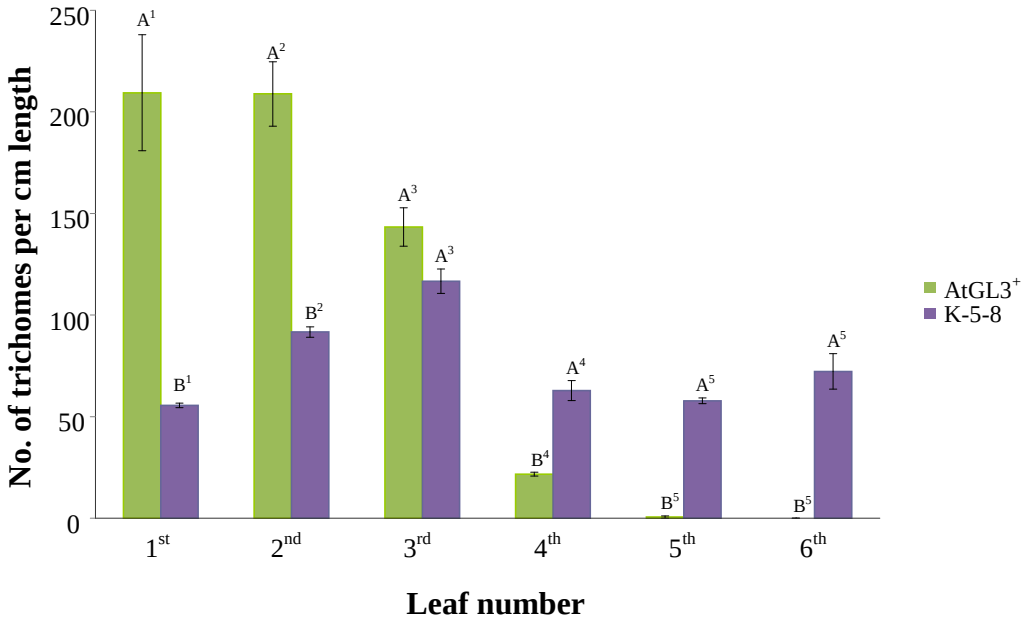


Fig. 2.21. Mean trichome densities along a 1 cm length of petiole randomly selected across the first-to-sixth leaves of AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*). Trichome densities were measured on first and second leaves from seedlings two WAG and third – sixth leaves from seedlings three WAG. A Tukey test was carried out between each of the plant lines at each leaf. Means of three plants per line ($\pm$ standard error) with same letters do not differ significantly ($P \leq 0.05$). Trichome densities were measured three times (i.e. on three separate positions along each petiole) on three independent plants per line. WAG – weeks after germination. Numbers in superscript indicate that there is no cross comparison between tissues and the comparison is only between lines.

Clustering of trichomes and trichome lengths were also recorded for leaf tissue for both lines. While the typical number of trichomes in a cluster was usually 2-3 for AtGL3$^+$ leaves, K-5-8 had clusters of up to 5 trichomes (**Fig. 2.22**). Clusters were present approximately two times more frequently in K-5-8 than AtGL3$^+$. Short aborted trichomes were also present on leaves of both plant lines, with a higher frequency on AtGL3$^+$ leaves (**Fig. 2.22 B,D**) than on K-5-8 leaves. Aborted trichomes were more frequent on AtGL3$^+$ leaves after the third leaf. Finally, trichomes

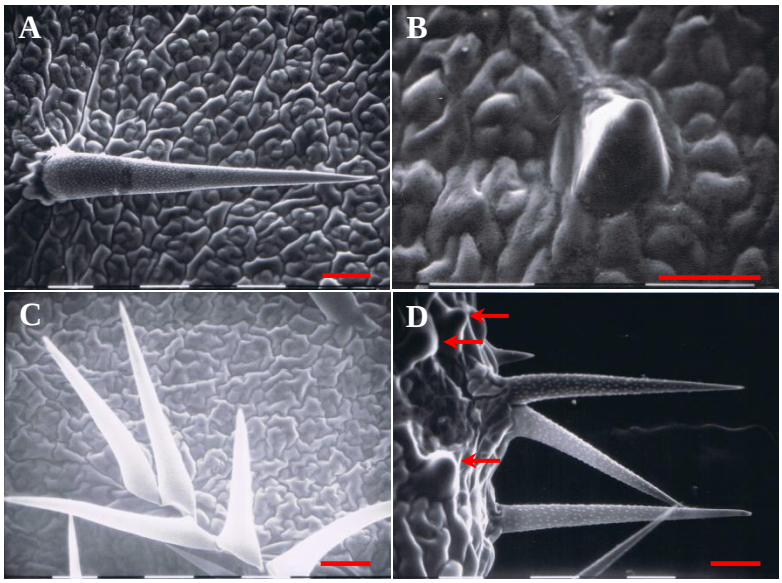


Fig. 2.22. Scanning electron micrographs showing different trichome morphologies on seedlings of K-5-8 and AtGL3$^+$. A) Typical fully developed single trichome of K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*); B) typical aborted trichome on AtGL3$^+$ *B. napus*; C) trichome cluster on K-5-8; D) trichome cluster and typical aborted trichomes (arrows) on AtGL3$^+$ *B. napus*. Size bar represents 0.1 mm.

on K-5-8 were consistently taller than those on AtGL3$^+$ plants, and the differences in length were approximately 150 µm throughout the series of trichomes measured (**Fig. 2.23**).

In summary, petioles and the abaxial surfaces of AtGL3$^+$ leaves had more trichomes than K-5-8 on the first three leaves after which, trichome densities declined on AtGL3$^+$ plants. However, the number of trichomes on the adaxial surface of all leaves was always higher on K-5-8 than AtGL3$^+$ plants (**Table 2.4**). Aborted trichomes were more frequent on AtGL3$^+$ leaves than K-5-8 leaves, where trichomes were also taller.

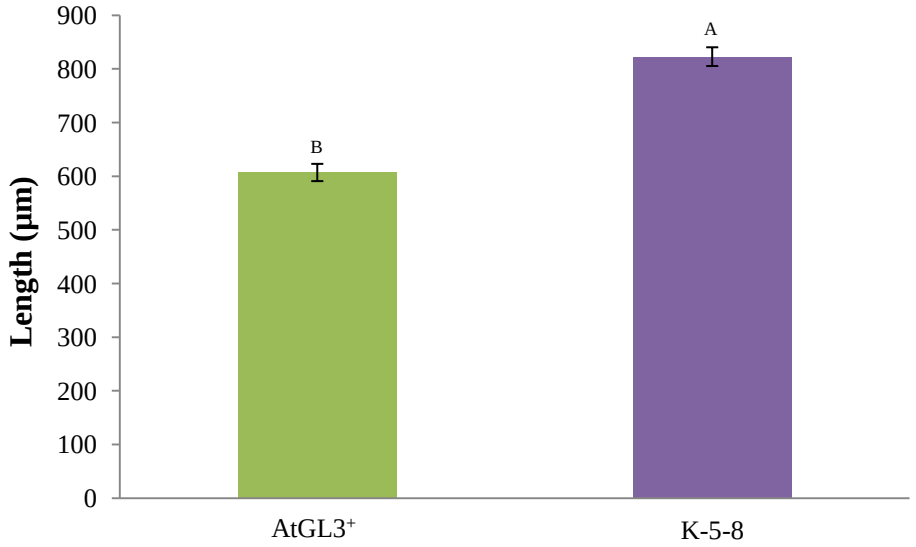


Fig. 2.23. Leaf trichome lengths on three week old seedlings of AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) measured using a ZEISS epifluorescent microscope. Trichome lengths were measured on first – fourth leaves of seedlings three weeks after germination. A Tukey test was carried out between the trichome lengths of each plant line. Means of 65 plants per line (± standard error) with different letters differ significantly ($P \leq 0.05$).

2.3.4.2. Agronomic characteristics of K-5-8 under a controlled greenhouse environment

The agronomic characteristics of three plant lines *B. napus* cv Westar, AtGL3^{+} *B. napus* and K-5-8, were measured for several growth parameters. Growth of the K-5-8 line was robust and similar to that for Westar when compared to AtGL3^{+} which showed a growth penalty during early developmental stages (Gruber *et al*., 2006). Growth of the O-3-7 line was not measured due to the length of time needed to develop a homozygous single insertion line.

The number of days from seeding for 100% germination, pre-bolting, bolt initiation and 1st flower initiation were measured for the three plant lines grown under a controlled greenhouse environment (**Fig. 2.24**). While there was no significant difference at $P \leq 0.05$ between these different growth events under the conditions tested, K-5-8 and AtGL3^{+} did show a trend towards significant difference (later in development) versus Westar for germination, bolting and flowering at $0.05 < P \leq 0.1$.

Table 2.4. Summary of mean trichome densities on edge, adaxial surface, abaxial surface and petiole of first-to-sixth leaves of AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*) lines.

Tissue	Plant line	Mean trichome density					
		1st leaf	2nd leaf	3rd leaf	4th leaf	5th leaf	6th leaf
Edge[1]	AtGL3$^+$	31.7 ± 0.9	29.4 ± 2.0	41.1 ± 1.1	10.0 ± 2.9	1.7 ± 0.9	0.6 ± 0.6
	K-5-8	41.7 ± 1.7	41.1 ± 3.1	36.1 ± 2.0	23.9 ± 2.0	25.6 ± 0.6	33.3 ± 0.0
Adaxial surface[2]	AtGL3$^+$	202.8 ± 7.3	75.0 ± 17	50.0 ± 0.0	13.9 ± 7.3	11.1 ± 7.3	1.1 ± 0.6
	K-5-8	288.9 ± 5.5	205.6 ± 5.5	280.6 ± 12	163.9 ± 7.3	177.8 ± 2.8	258.3 ± 9.6
Abaxial surface[1]	AtGL3$^+$	98.3 ± 0.9	75.0 ± 8.2	120.6 ± 13	20.0 ± 0.9	6.7 ± 0.9	1.1 ± 0.6
	K-5-8	44.4 ± 1.5	58.3 ± 5.4	68.9 ± 5.6	65.0 ± 2.9	63.9 ± 3.1	59.4 ± 7.1
Petiole[1]	AtGL3$^+$	209.4 ± 28	208.9 ± 16	143.3 ± 9.5	21.7 ± 0.9	0.6 ± 0.6	0.0 ± 0.0
	K-5-8	55.6 ± 1.1	91.7 ± 2.5	116.7 ± 6.0	62.8 ± 4.9	57.8 ± 1.5	72.2 ± 8.7

[1] Mean trichome densities along a 1 cm length

[2] Mean trichome densities on 1 cm^2 area

± Standard error of means

Plant height was also measured at weekly intervals for six weeks after seeding (**Fig. 2.25**). For the first two weeks, *B. napus* cv Westar plants were the tallest, followed by K-5-8 and AtGL3$^+$ plants, respectively. Plant height of *B. napus* cv Westar was significantly taller than that of AtGL3$^+$ plants for the first three weeks ($P \leq 0.05$), while K-5-8 was initially shorter than Westar and then similar to Westar by two weeks through to the five week period ($P \leq 0.05$). By the 6th week K-5-8 was significantly taller than Westar, while the AtGL3$^+$ plants lagged behind both Westar and K-5-8 over the entire growth period except when height variability was high for Westar at the fourth week.

There was no difference between the shape or size of seeds of Westar and K-5-8. However, AtGL3+ seeds were comparatively smaller than the other two lines. Total seed weight of *B. napus* cv Westar, AtGL3$^+$ and K-5-8 grown from November to February under controlled

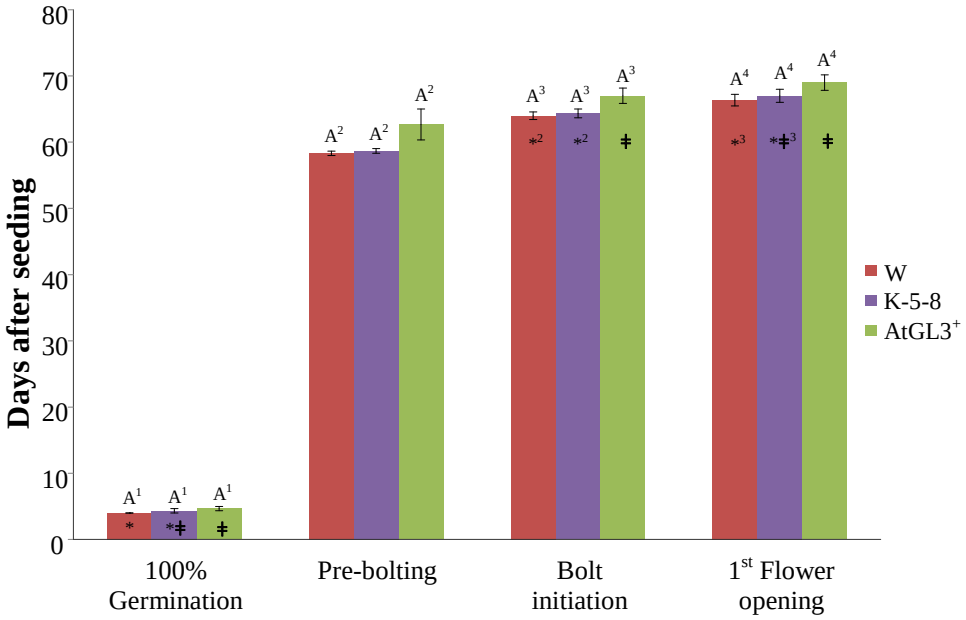


Fig. 2.24. Onset of developmental stages for *B. napus* cv Westar (W), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) lines under greenhouse conditions. Tukey test was carried out between each of the plant lines at each growth stage. Means of three plants per line (± standard error) with same letters do not differ significantly at $P \leq 0.05$. Those means showing a trend towards significant difference at $0.05 < P \leq 0.1$ are indicated (*, ǂ). Measures were carried out on three independent plants per line. Developmental stages were evaluated twice weekly. Pre-bolting – onset of flower buds; bolt initiation – appearance of peduncle; first flower opening – initial opening of flowers. Numbers in superscript indicate that there is no cross comparison between developmental stages and the comparison is only between lines.

greenhouse conditions was measured to see the effect of the transgene on final seed yield (**Fig. 2.26**). The highest mean total seed weight of 18.72 g per plant was obtained for Westar, however it was not significantly different ($P \leq 0.05$) to that for K-5-8, which had a mean total seed weight of 17.99 g. Total mean seed weight for AtGL3^{+} plants was significantly lower than both Westar and K-5-8 at 15.01 g (**Fig. 2.26**).

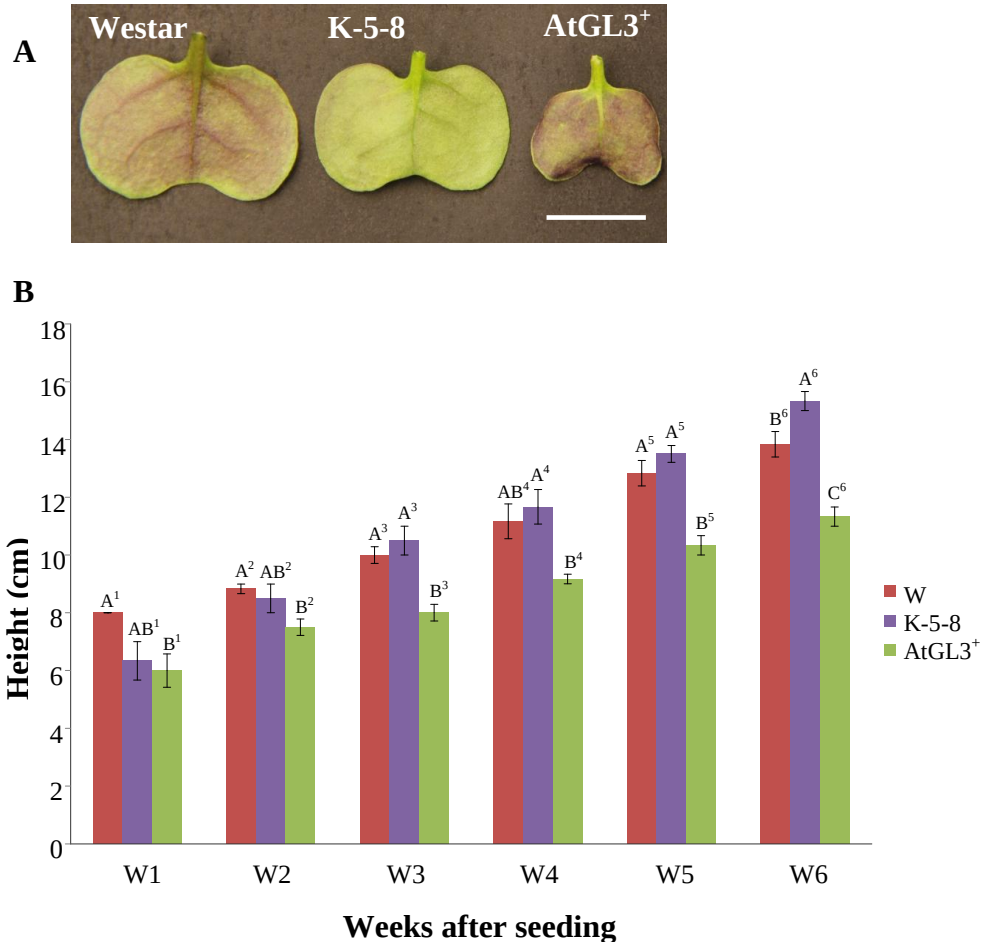


Fig. 2.25. Cotyledon size and seedling growth (plant height) of *B. napus* cv Westar (W), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A) Comparative size of cotyledon abaxial surfaces of five-day-old *B. napus* seedlings grown under 24 h continuous light in a growth chamber. Note: *B. napus* cv Westar showing light-to-medium purple colouration; K-5-8 showing no purple colouration and AtGL3^{+} *B. napus* showing the strongest purple colouration. B) Plants were grown over a period of six weeks under controlled greenhouse conditions from November to February in 2010/2011, Saskatoon. A Tukey test was carried out between each of the plant lines at each week. Means of three plants per line (± standard error) with same letters do not differ significantly ($P \leq 0.05$). Measurements were carried out on three independent plants per line. Numbers in superscript indicate that there is no cross comparison between weeks after germination and the comparison is only between lines. Size bar represents 1 cm.

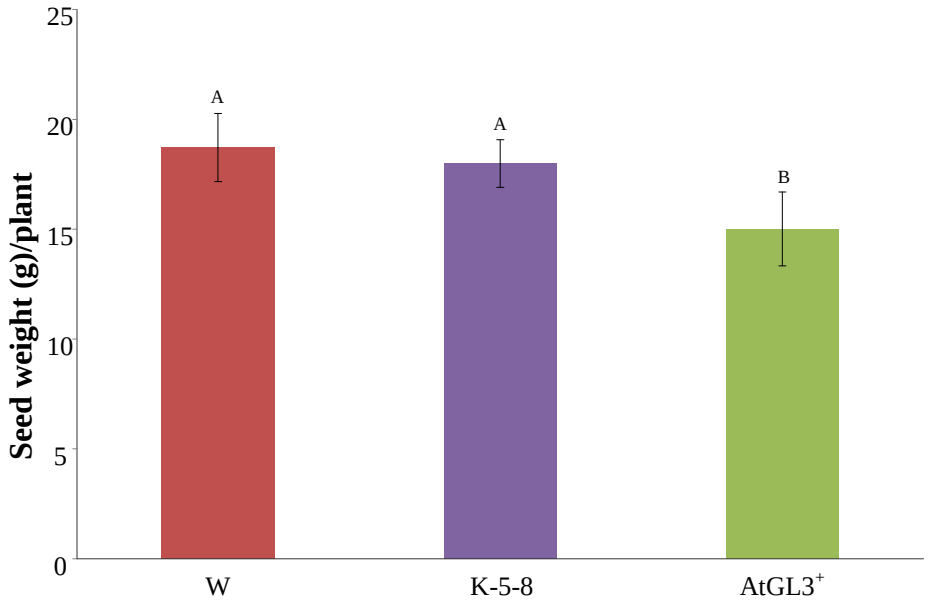


Fig. 2.26. Total seed weight (g) per plant of *B. napus* cv Westar (W), AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*) under controlled greenhouse conditions in February, 2011, Saskatoon. A Tukey test was carried out between the seed weights of the plant line. Means of three plants per line (± standard error) with same letters do not differ significantly ($P \leq 0.05$). Measurements were carried out on three independent plants per line.

Hundred seed weight was also measured for the three plant lines (**Fig. 2.27**). While K-5-8 had the highest numerical weight at 0.41 g / 100 seeds and AtGL3$^+$ with 0.36 g / 100 seeds had the lowest, there was no significant difference ($P \leq 0.05$) between 100 seed weight of the three lines tested under greenhouse conditions.

2.3.5. Expression pattern of primary trichome regulatory genes

The transformation of *B. napus* cv Westar with *Arabidopsis 35S:GL3* (Gruber *et al*., 2006) produced the AtGL3$^+$ line with a dense cover of trichomes on seedling tissues but with a smaller stature compared with Westar. A subsequent re-transformation of this AtGL3$^+$ line with the RNAi K-TTG1 construct produced the K-5-8 line with restored growth and a broader coverage of trichomes on seedling tissues. A similar transformation of AtGL3$^+$, but with the over-expression O-TTG1 construct, resulted in a taller stature, reduced trichome density, poor

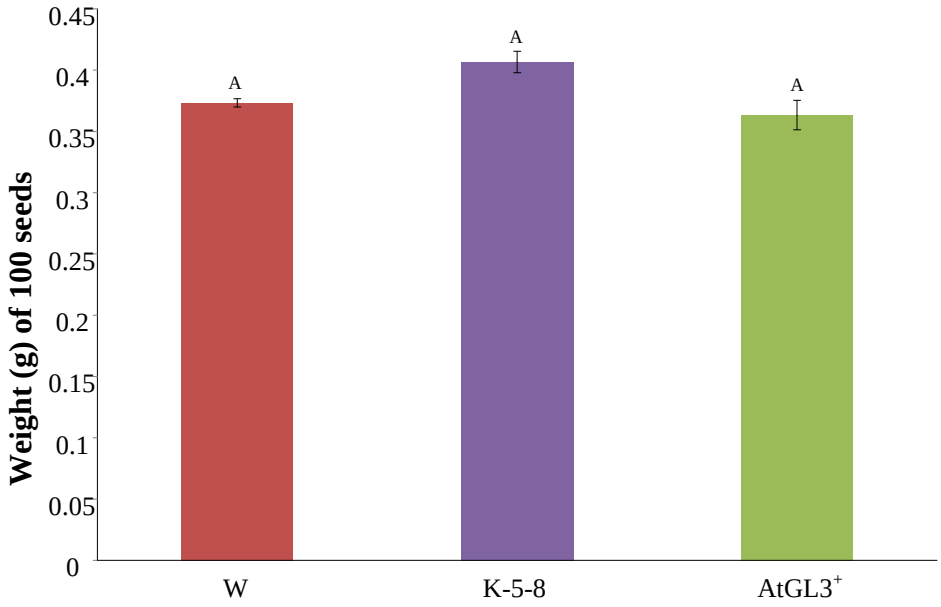


Fig. 2.27. Weight (g) of 100 seeds of *B. napus* cv Westar (W), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under greenhouse conditions. A Tukey test was carried out between seed weights of each of the plant lines. Means of three seed lots from three independent plants per line ($\pm$ standard error) with same letters do not differ significantly ($P \leq 0.05$).

seed yield and frequent plant death. The relationship between transcript levels of the original *Arabidopsis 35S:GL3* and the introduced *BnTTG1* together (as members of MBW tri-protein complex) may be affecting the transcript levels of other trichome initiation / development regulatory genes in these lines. Hence, expression of five *B. napus* trichome regulatory genes related to the complex (*GL1*, *GL2*, *GL3*, *TTG1* and *TRY*), as well as *AtGL3* and *B. napus ACT2*, was measured in glabrous cotyledons, 1st-3rd leaves and 4th-6th leaves of K-5-8, O-3-7, AtGL3^{+} and Westar using qRT-PCR.

Primers were designed using available *B. napus* cDNA and EST sequences as well as *Arabidopsis* sequence data (see **Appendix 6.2** primer coverage). Full length cDNA sequences were available for each of the known copies of the *B. napus GL1* (1), *GL2* (2) and *TTG1* (2) orthologs (www.ncbi.nlm.nih.gov) and an EST sequence was also available for the *B. napus TRY* ortholog (www.brassicagenomics.ca). Using Arabidopsis *GL3* cDNA specific primers, a ~500 bp

BnGL3 product was amplified using *B.napus* cDNA as a template. Conserved regions between the *B. napus* gene family members were used to design qRT-PCR primers (**Table 2.2**). Transcript levels of the trichome stimulating *B. napus* orthologs of *GL1, GL2, GL3* and *TTG1* genes and the inhibitory *TRY* as well as *AtGL3* are shown relative to transcript levels in Westar cotyledons **(Figure 2.28)**. *B. napus BnACT2* was used as an internal control and was normalized to the expression of *ELONGATION FACTOR 1* (*BnEF1*). There was no significant difference ($P \leq 0.05$) between the relative expressions of *BnACT2* in all three tissues of Westar, $AtGL3^+$, K-5-8 and O-3-7 (**Fig. 2.28A**). However, a high variability was observed between the three sample plants of each line.

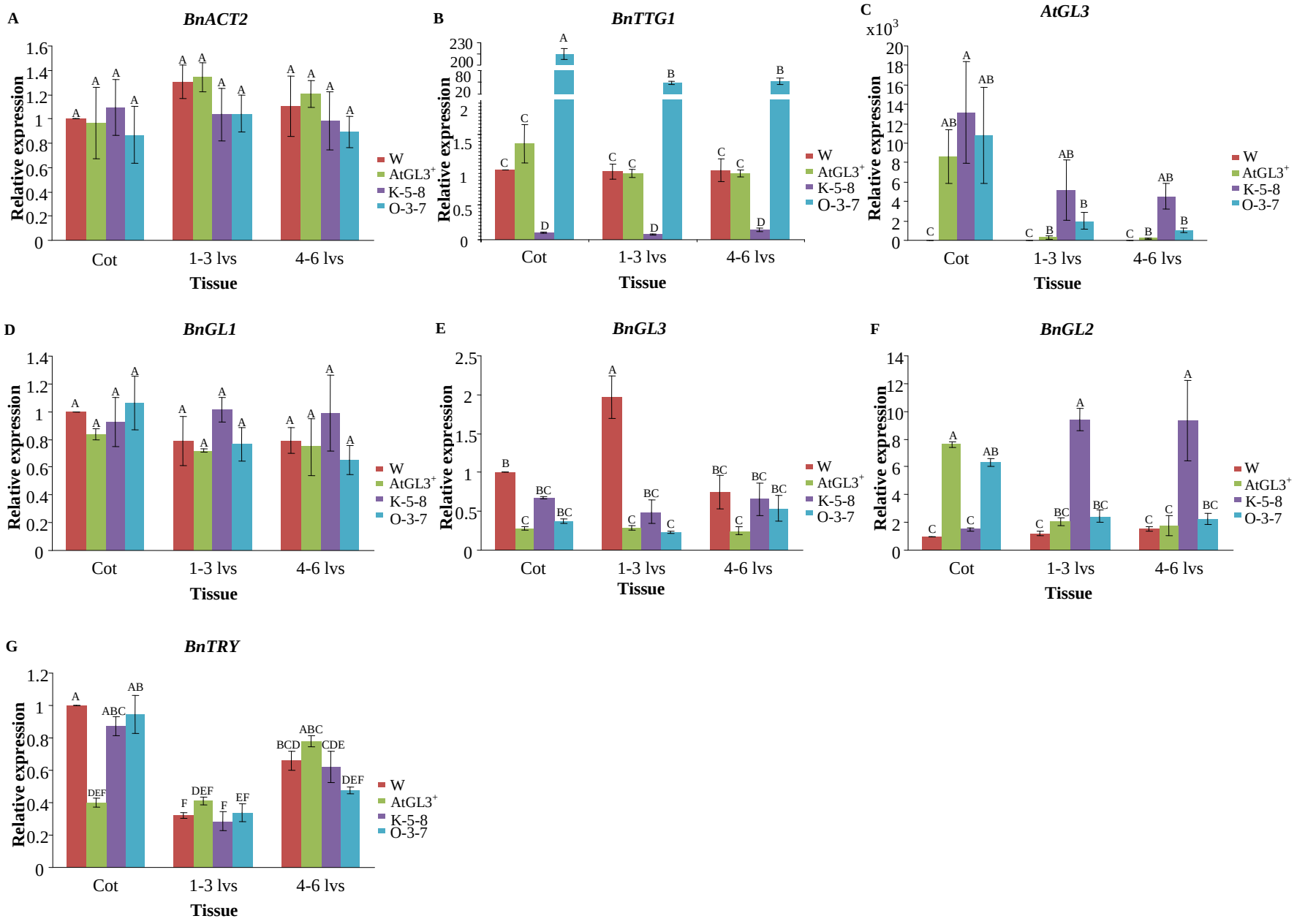
As expected, relative *BnTTG1* transgene expression in O-3-7 was significantly higher ($P \leq 0.05$) in all tissues (cotyledons and leaves) than in the three other lines and this expression was between 80-200-fold higher than in Westar tissues (**Fig. 2.28B**). Line K-5-8 showed the lowest *BnTTG1* expression in all three tissues compared with the other lines, and was 50-100-fold lower than in Westar. There was no significant difference between the expression of *BnTTG1* in any of the tissues of Westar or $AtGL3^+$.

High variability in relative expression levels of the *AtGL3* transgene was observed in K-5-8, $AtGL3^+$ and O-3-7 lines for cotyledons and for leaves of $AtGL3^+$ plants (**Fig. 2.28C**). Highest expression of *AtGL3* for all three transgenic lines was detected in cotyledons followed by 1-3 leaves and 4-6 leaves. A similar numerical pattern between lines was detected in all three tissues with the highest level in K-5-8 followed by O-3-7 and $AtGL3^+$.

There was no significant difference ($P \leq 0.05$) in relative expression of *BnGL1* between any of the lines for the three tissues tested nor between the three tissues of each line (**Fig. 2.28D**).

The highest relative expression of *BnGL3* was found in the glabrous first three leaves of Westar. The level was 2-fold higher ($P \leq 0.05$) than the rest of the Westar tissues and even higher than that for each of the three tissues of K-5-8, $AtGL3^+$ and O-3-7 (**Fig. 2.28E**). The three tissues of $AtGL3^+$ and O-3-7 showed the lowest *BnGL3* expression levels followed by K-5-8. Especially for all tissues of the $AtGL3^+$ line, the *BnGL3* expression level remained consistently the lowest of all lines and had the lowest variability.

Fig. 2.28. Relative transcription of *GL1*, *GL2*, *GL3*, *TTG1* and *TRY B. napus* orthologs and *AtGL3* in seedling tissues of *B. napus* cv Westar (W), line K-5-8 (*BnTTG1* knock-down in $AtGL3^+$ *B. napus*), line O-3-7 (*BnTTG1* over-expressed in $AtGL3^+$ *B. napus*) and $AtGL3^+$ *B. napus* ($AtGL3^+$) using qRT-PCR. Panel (A); Expression of *ACT2* control gene in cotyledons of glabrous *B. napus* cv Westar (set at 1) was normalized to the expression of the constitutive *B. napus* 'housekeeping' gene *EF1*. Panels (B-F); Expression of individual trichome genes is relative to their level in cotyledons of glabrous *B. napus* cv Westar (set at 1), which was normalized to the expression of the *B. napus* gene *ACT2*. A Tukey test was carried out to compare mean relative expression of each gene on the four plant lines and between different tissues. Means of three plants per line ($\pm$ standard error) with same letters do not differ significantly ($P \leq 0.05$). Relative expression was measured on one RNA sample each from three independent plants per line. Three technical replicates were used for each RNA sample.


A
BnACT2
B
BnTTG1
C
AtGL3
x10^3
D
BnGL1
E
BnGL3
F
BnGL2
G
BnTRY
Relative expression
Tissue
Cot
1-3 lvs
4-6 lvs
W
AtGL3+
K-5-8
O-3-7

For the remaining trichome stimulatory gene, *BnGL2* (which is affected by the MBW in Arabidopsis), Westar showed the lowest expression level of all four lines and in all three tissues (**Fig. 2.28F**). In cotyledons, AtGL3+ and O-3-7 had the highest *BnGL2* relative expression and it was significantly different ($P \leq 0.05$) from both Westar and K-5-8. However, in 1^{st}-3^{rd} and 4^{th}-6^{th} leaves, the pattern was reversed. Even though variability increased in hairy K-5-8, this line showed the highest relative expression, while the glabrous O-3-7, hairy AtGL3$^+$ and glabrous Westar lines all had similar low expression levels.

For the *TRY* gene (which is a negative regulator induced by the MBW complex in Arabidopsis), glabrous cotyledons showed the highest *BnTRY* relative expression compared to the other two tissues with the exception of AtGL3$^+$ (**Fig. 2.28G**). There was no significant difference between cotyledon *BnTRY* relative expression in the other three lines ($P \leq 0.05$), while the *TRY* was 2-fold lower in AtGL3$^+$ cotyledons. A similar low expression level of *BnTRY* was detected in the first three leaves of each line, whether hairy (in the case of AtGL3$^+$ or K-5-8) or glabrous (in the case of Westar and O-3-7). Relative expression levels increased somewhat equally in 4^{th}-6^{th} leaves of all four lines when compared to 1-3 true leaves.

2.3.6. Anthocyanin accumulation, expression of primary anthocyanin regulatory genes and seed mucilage analysis

To study the roles of *AtGL3* and *BnTTG1* in anthocyanin accumulation in *B. napus*, Westar, AtGL3$^+$ and K-5-8 plants were grown under 24 h continuous white light and 22^{o}C. After 10 days, the abaxial-surface of Westar cotyledons showed a light-to-medium purple colouration. AtGL3$^+$ cotyledons had the strongest purple colouration and K-5-8 cotyledons had no purple colouration (**Fig. 2.25A**).

Anthocyanin content in the above-ground seedling tissues of these plants corresponded to this colouration. AtGL3$^+$ plants accumulated the highest anthocyanin content (5.5 mg/g) which was significantly higher ($P \leq 0.05$) than that of both Westar and K-5-8 (**Fig. 2.29**). The lowest anthocyanin accumulation of 2.64 mg/g FW, which was significantly lower than Westar, occurred in K-5-8 seedlings.

Since plants transformed with both *Arabidopsis GL3* and K-TTG1 showed a distinct reduction in anthocyanin accumulation, it was proposed that these two gene inserts were affecting the transcript levels of anthocyanin pathway genes. Expression levels of the *B. napus*

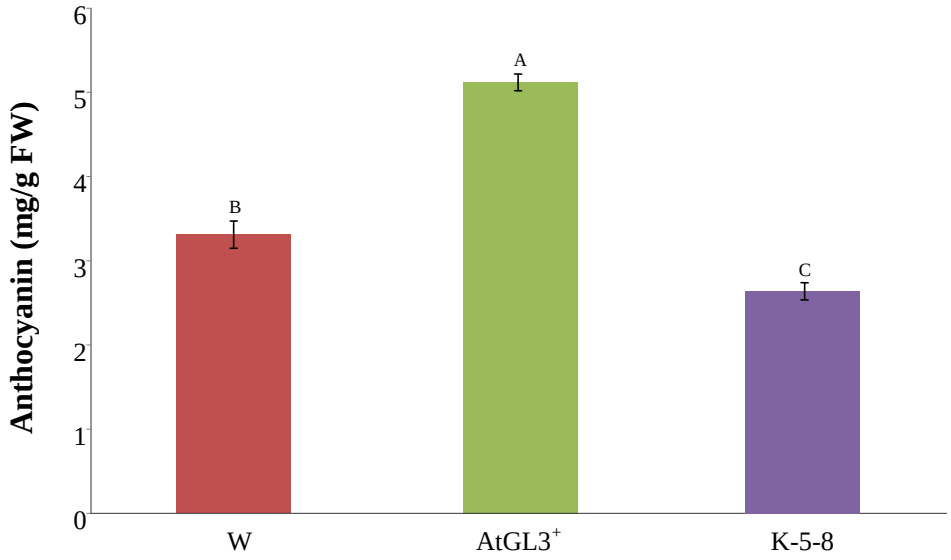


Fig. 2.29. Total seedling anthocyanins accumulated in *B. napus* cv Westar and transgenic plants grown under 24 h continuous light and temperature. Anthocyanins were extracted from 10-day-old above ground tissues of *B. napus* cv Westar (W), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) and quantified against a standard curve for cyanidin chloride. A Tukey test was carried out between total anthocyanins of each of the plant lines. Means of three plants per line ($\pm$ standard error) with different letters differ significantly ($P \leq 0.05$). Total anthocyanins were measured on three biological replicates each consisting of three independent plants per line. FW- fresh weight.

BnANS in AtGL3^{+} was over 10-fold higher than that of K-5-8, which in turn was significantly lower ($P \leq 0.05$) than that in Westar. Similarly, *BnGST* expression level in AtGL3^{+} was 15-fold orthologs of three anthocyanin pathway genes in Arabidopsis, namely *BnANTHOCYANIDIN SYNTHASE* (*BnANS*), *BnDIHYDROFLAVONOL 4-REDUCTASE* (*BnDFR*) and *BnGLUTATHIONE S-TRANSFERASE* (*BnGST*), were measured using qRT-PCR in all three lines (**Fig. 2.30**).

The highest relative expression of all three genes tested was observed in AtGL3^{+} seedlings, with the lowest level in K-5-8 seedlings **(Fig. 2.30)**. These expression levels support

the anthocyanin accumulation pattern observed for these lines **(Fig. 2.29)**. Relative expression of higher than Westar which in turn was two-fold higher than that of K-5-8 **(Fig. 2.30)**. In contrast, *BnDFR* expression levels in Westar and K-5-8 were not significantly different, while AtGL3^{+} seedlings had a 6-fold higher level of *BnDFR* ($P \leq 0.05$) than both Westar and K-5-8.

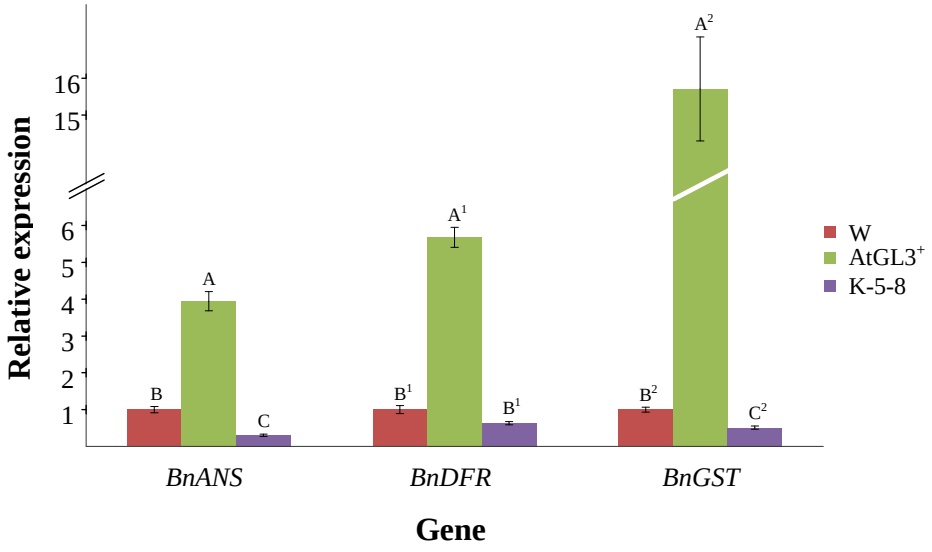


Fig. 2.30. Quantitative RT-PCR of the anthocyanin biosynthetic genes *BnANS*, *BnDFR* and *BnGST* in seedlings of *B. napus* cv Westar (W), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) grown under 24 h light in a growth chamber. Expression of individual genes are relative to that measured in glabrous *B. napus* cv Westar (set at 1), which has been normalized to the expression of the *B. napus* gene *ACT2*. A Tukey test was carried out for the relative expression of each gene. Means of three plants per line (± standard error) with the same letters do not differ significantly ($P \leq 0.05$). Relative expression was measured on one RNA sample each from three independent plants per line. Three technical replicates were used for each RNA sample.

Since in Arabidopsis, modification of *TTG1* expression affects seed mucilage, seed mucilage of *B. napus* cv Westar, AtGL3^{+} and K-5-8 was determined using a histochemical protocol modified from *Camelina sativa* seed mucilage screening. No seed mucilage was

observed on any of the *B. napus* lines tested with both India ink and Ruthenium red stains. Seed mucilage of Camelina seeds was visible with both stains (**Fig. 2.31A,B**).

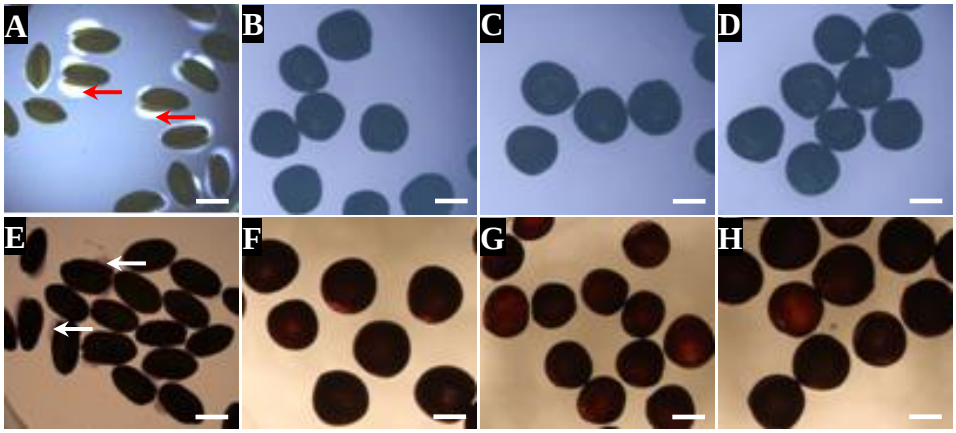


Fig. 2.31. Seed mucilage screening of field harvested seeds of *B. napus* cv Westar, AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A - D) Stained with India ink. E - H) Stained with Ruthenium red. A,E) Wild-type *Camelina sativa* showing seed mucilage (arrows). B,F) *B. napus* cv Westar. C,G) AtGL3^{+}. D,H) K-5-8. Size bar represents 1 mm.

2.4. Discussion

2.4.1. *TTG1* affects *B. napus* transformation and growth in the presence of *AtGL3* gene

The effect of the *Brassica napus TTG1* gene on growth and seed yield was examined by manipulating *TTG1* dosage in Westar and AtGL3^{+} *B. napus* backgrounds. Transformation in Westar for both over-expression and knock-down constructs of the *BnTTG1* gene resulted in a 2-fold higher number of transformants when compared to transformation in the AtGL3^{+} *B. napus* line. The original transformation of *B. napus* cv Westar with the AtGL3^{+} construct had also resulted in very few transgenic plants, and those that had survived showed severe changes in growth and fertility in early generations (Gruber *et al*., 2006). The most advanced homozygous AtGL3^{+} single insertion *B. napus* line used in this thesis as a background line to develop the

BnTTG1 over-expression or knock-down lines also continued to show the same modified growth phenotypes, such as short stature with slow vegetative and reproductive growth and curled leaves, although they were not as extreme as earlier generations. These phenotypes could be the result of the pattern of expression of *AtGL3,* particularly under the control of the 35S promoter instead of a *GL3* promoter. Less extreme growth abnormalities were observed in AtGL3^{+} *B. napus* plants with a single TDNA insertion over multiple TDNA insertions (Gruber *et al*., 2006). Similar growth and morphology abnormalities were previously reported for Arabidopsis plants over-expressing *AtGL2* under the control of the 35S promoter (Ohashi *et al*., 2002) suggesting that at least two trichome genes (*GL3* and *GL2*) can affect both growth and trichome patterning depending on their expression profile. However, these abnormal phenotypes could also be due to gene disruption at the site of TDNA insertion. In AtGL3^{+} *B. napus* plants, the site of T-DNA insertion has not been identified.

Transformation of a *B. napus* cv Westar background with either O-TTG1 or K-TTG1 did not produce an abnormal growth or trichome phenotype compared with the phenotype of untransformed Westar plants even though the relative expression of *BnTTG1* varied from a 10-fold decrease in knocked-down lines to an 80-fold increase in over-expressed lines. This outcome suggests that altered *TTG1* expression alone is not sufficient to produce growth or trichome phenotypic differences in this background. Moreover, the transformation efficiency with these *TTG1* constructs was unaffected in the Westar background compared with efficiencies using other transgenes, such as an empty vector control tested in this work (data not shown).

Transformation of the AtGL3^{+} *B. napus* background with K-TTG1 produced more viable transformants than transformation with O-TTG1, which was severely affected by the latter construct. Out of seven confirmed over-expression transformants, only line O-3 (with a single insert) produced seeds. The multiple insertion line O-9 also flowered, but was unable to produce pods. In contrast, five out of nine confirmed K-TTG1 transformants produced seed and four out of the five fertile plants had single insertions. All fertile K-TTG1 transformants showed normal growth and development. The abnormal developmental phenotype of the over-expressing lines was probably the result of over-expression of *BnTTG1* from the *35S* promoter in the presence of the *AtGL3* gene, with the mildest phenotype in the only plant that set seed and that had a single rather than multiple TDNA insertion(s). Since this abnormal growth phenotype was not observed

in *B. napus* cv Westar plants over-expressing *BnTTG1*, the data suggests that the resulting phenotype in the O-TTG1 plant was due to an *AtGL3*/*TTG1* interaction.

The role of *TTG1* and the *bHLH GL3* in plant growth has so far been studied in very little detail. Arabidopsis plants expressing *35S:GL1* together with the maize *bHLH R* gene show seedling lethality; however, *35S:GL1*/*35S:R*-GR plants (*35S:GL1* plants containing an inducible form of the *R* gene) are viable in the absence of the inducer, and the most vigorous of these plants are also homozygous for a *ttg1* mutation (Szymanski *et al*., 1998). These latter reports suggest that Arabidopsis plant viability is also sensitive to *TTG1* and *bHLH* dosage and that both gene types have a role in normal plant growth and viability. Transformation data in this thesis suggests a similar role for *TTG1* in *B. napus*, since a knock-down of *TTG1* in the presence of *AtGL3* produced more vigorous *B. napus* plants than AtGL3$^+$ *B. napus* lines on their own. Over-expression of *TTG1* in O-3-7 produced spindly plants with greatly reduced seed set, and reproductive ability was completely eliminated in other multiple *TTG1* insertion *B. napus* over-expression lines in the AtGL3$^+$ background. Hence, over-expression of *bHLH* genes in general may have an adverse effect on viability and growth at least in the Brassicaceae.

GL3 and *TTG1* are also involved in root hair patterning, therefore, these extreme growth phenotypes could be due to an effect on root growth. However, experiments comparing *B. napus* cv Westar, AtGL3$^+$, K-5-8 and O-3-7 to determine the effect of *GL3* and *TTG1* on root growth did not result in data sufficiently consistent to include in this thesis.

2.4.2. *TTG1* affects trichome patterning in *B. napus* in the presence of the *AtGL3* gene

Of the lines generated by transformation using K-TTG1 and O-TTG1 in the hairy AtGL3$^+$ *B. napus* background, only O-3-7 (the only surviving *BnTTG1* over-expression line) and K-5-8 and K-6-3 (both single *BnTTG1* TDNA insertion lines) were developed into homozygous lines. Lines with K-TTG1 and O-TTG1 in the glabrous Westar background were not advanced to homozygous lines during this project's research since their growth and trichome phenotype remained identical to Westar and greenhouse space was limited. O-3-7 showed a dramatically decreased trichome density phenotype compared to its "hairy" parental line AtGL3$^+$ and had very few trichomes during vegetative growth, suggesting that over-expression of *BnTTG1* in the AtGL3$^+$ *B. napus* background was able to restore a wild type trichome density phenotype. In contrast, K-5-8 (preferred for detailed analysis over K-6-3) showed the most extreme enhanced

trichome phenotype compared with the AtGL3^{+} background, although K-6-3 also was similarly enhanced. K-5-8 had larger trichomes and expanded trichome density up to the 12th leaf compared to the background over-expression line of *AtGL3* in *B. napus* cv Westar, which showed increased trichomes only on the first three leaves, decreasing on the fourth leaf, and resembling wild-type by the sixth leaf (Gruber *et al*., 2006). The increased trichome density phenotype on later leaves of K-5-8 suggests a role for *TTG1* in trichome formation on later leaves in AtGL3^{+} background. This impact of *TTG1* transcript reduction on *B. napus* later leaf patterning should be tested in other types of Brassica plants in which trichomes are found on leaves at later development stages such as in *B. villosa* (Palaniswamy and Bodnaryk, 1994) and *B. nigra* (Traw and Feeny, 2008). In other Brassica species and Arabidopsis, expansion of trichome patterning on later leaves has not been seen in any known mutation.

In this *B. napus* thesis study, the adaxial surface of the leaf and the petiole of K transgenics had the highest number of trichomes when compared to the leaf abaxial surface and margin. Different tissues or tissue layers/areas may have different capabilities to respond to trichome initiation signals. The leaf adaxial epidermis and petiole may be more sensitive to a trichome initiation signal than the abaxial surface or leaf margin, which have fewer trichomes. Trichome formation is suppressed in Arabidopsis by single-repeat R3MYBs in a tissue-specific, highly redundant manner. For example, trichome formation on the Arabidopsis petiole is redundantly suppressed by TRY, CPC and ETC2, whereas trichomes on the inflorescence are suppressed by CPC and TCL1 (Wang *et al*., 2008). Within Arabidopsis leaves, trichome initiation genes also have differential impacts on different tissue types. For example, *gl1*, *ttg1* and *gl2* mutant lines produce fewer trichomes on the leaf mid-vein region while both *gl1* and *ttg1* lines maintain wild type levels of leaf margin trichomes, and *gl2* leaf margin trichomes are less branched than wild type trichomes (reviewed in Marks, 1997).

In Arabidopsis, trichome initiation/density appears to be regulated for individual organs, with usually no trichomes present on hypocotyls, cotyledons, petals, stamens or carpels (Szymanski and Marks, 1998). Organ patterning also varies with trichomes in the Brassicaceae, but usually cotyledons are bare of trichomes, although recently trichomes have been discovered on the abaxial cotyledon surface of a range of *Sinapis alba* germplasm (Taheri, Nayidu and Gruber, unpublished). Consistent with Arabidopsis and most Brassicas, no trichomes were observed on cotyledons of any of the three *B. napus* transgenic lines tested in this study. The

development of trichomes on cotyledons may be regulated differently than on leaves. In Arabidopsis, a mutation *lec1* in the HAP3 B-domain CCAAT binding factor *LEAFY COTYLEDON* results in formation of trichomes on the adaxial surface of the cotyledons, but in this case the effect is due to a disruption in embryogenesis control (West *et al*., 1994). In terms of direct influence of trichome initiation genes, ectopic trichomes were found on cotyledons of *35S:AtMYB23* over-expression lines developed by transforming a wild type background, but not when transforming a *ttg1* background, suggesting that the balance between *TTG1* and this *R2R3MYB* gene is important for trichome formation on cotyledons (Kirik *et al*., 2001). In Arabidopsis, with other genes making up the traditional MBW complex, over-expression of the *R2R3 MYB* gene *GL1* also resulted in a few ectopic trichomes on cotyledons in a wild type background (Larkin *et al*., 1994). However, introduction of *AtGL1* into *B. napus* did not result in cotyledon trichome patterning in a previous study (Gruber *et al*., 2006). Moreover, in this thesis research, expression of *BnGL1* was virtually identical in cotyledons or leaves of over-expression and knock-down lines of *BnTTG1* in AtGL3$^+$ background. These data suggest that *BnGL1* is not regulated by *TTG1* in *B. napus* to affect cotyledon or leaf trichomes. Since no difference in transcript levels occurred for either *BnTTG1* or *BnGL1* in AtGL3$^+$ *B. napus* cotyledons and leaves compared to Westar, neither *GL1* nor *TTG1* in *B. napus* appear to be regulated by *AtGL3*, at least not directly.

2.4.3. *TTG1* and *AtGL3* affect trichome length (outgrowth) in *B. napus*

The trichomes on K-5-8 were taller than those on AtGL3$^+$ *B. napus* and the gene expression pattern suggested the balance between *TTG1* and *GL3* regulates trichome size (outgrowth). In Arabidopsis, trichome size was arrested in *gl2* mutants and shortened aborted trichome cells that were expanded laterally along the leaf surface were produced. Thus the Arabidopsis model suggests that increased expression of *GL2* is required for proper outgrowth of trichomes (Rerie *et al*., 1994). In *B. napus*, the higher expression of *BnGL2* in 1st-3rd and 4th-6th leaves of K-5-8 compared to that of the AtGL3$^+$ line correlates with enhanced trichome length in K-5-8. However, the aborted trichomes on AtGL3$^+$ *B. napus* leaf surfaces were not expanded laterally along the leaf surface as in Arabidopsis; instead their growth was arrested immediately after initiation. Moreover, there was a trend towards increased levels of *AtGL3* transcripts in K-5-8 compared to the AtGL3$^+$ line which may have an effect on trichome length. In the

Arabidopsis *gl3* mutant, trichomes are also smaller compared to wild type lines (Hulskamp *et al*., 1994).

Mutation in the Arabidopsis *TTG1* gene affects the level of the Arabidopsis *GL2* promoter activity, such that expression of *GL2* is reduced in Arabidopsis when *TTG1* transcription is reduced (Hung *et al*., 1998). However, expression of a *bHLH 35S:R* construct in Arabidopsis is sufficient to induce *GL2* promoter activity in the absence of TTG1 (Hung *et al*., 1998). Similarly, *GL3* transcripts can bypass the requirement in Arabidopsis for *TTG1* expression to induce the *GL2* promoter activity (Hung *et al*., 1998). However, in this *B. napus* study, a depressive effect was observed on *BnGL2* transcription by increasing *TTG1* transcript levels in leaves of line O-3-7 and a stimulatory effect by reducing *TTG1* transcripts. These data suggest that there may be two mechanisms for controlling *GL2* expression; i) TTG1-dependent in Arabidopsis and *B. napus* and ii) TTG1-independent in Arabidopsis.

A role for other genes, e.g., *GL1*, in *GL2*-dependent expression of trichome out growth in Arabidopsis shoots has also been proposed (Szymanski *et al*., 1998). GL3 can activate *GL2* expression of Arabidopsis trichome outgrowth in a GL1-dependent manner (Morohashi *et al*., 2007). Since *GL1* expression was unaltered in all four *B. napus* lines in this study, there may be a GL1-independent regulation of *GL2* in the control of *B. napus* trichome length. Similarly, *AtMYB23* affects *AtGL2* in Arabidopsis, but unlike the pattern of *GL1* expression in Arabidopsis, *AtMYB23* expression does not decline in fully developed trichomes suggesting that *AtMYB23* regulates the expression of *GL2* after *GL1* ceases its expression (Kirik *et al*., 2001). *AtMYBL2* also negatively regulates Arabidopsis *GL2* expression (Sawa, 2002). By binding to GL3, the single-repeat AtMYBL2 protein is proposed to interfere with the ability of GL1 to regulate *GL2* (Sawa, 2002). With such multi-regulation of trichome outgrowth and *GL2* in Arabidopsis, these additional genes should all be tested for their expression pattern and role in trichome outgrowth in the new *B. napus* lines.

In Arabidopsis, TTG2 function overlaps that of GL2 in the control of trichome outgrowth (Johnson *et al*., 2002). Using gene expression analysis and ChIP experiments, Zhao *et al*. (2008) reported that Arabidopsis *TTG2* is a direct target of TTG1 and GL3. These observations plus those presented above suggest that the regulation of trichome out growth (at least in Arabidopsis) is a complex process involving several counteracting factors rather than a simple linear process.

For example, in Arabidopsis, a mutation in *GT-2-LIKE1* (*GTL1*) results in increased trichome cell size without altering the overall surface patterning or branching of the trichome (Breuer *et al*., 2009). Expression of this negative regulator, *GTL1*, is largely dependent on *TTG1* and *GL2*, although these two factors are not likely to be the only requirement for *GTL1* expression (Breuer *et al*., 2009). *GTL1* and *TTG2* expression should now be tested in K-5-8 for their role in trichome outgrowth in *B. napus*. A low expression of *GTL1* would be consistent with taller trichomes in K-5-8 compared to those of AtGL3^{+} *B. napus*.

2.4.4. Comparison of the *Brassica napus* leaf trichome initiation genes with the Arabidopsis MBW model

In Arabidopsis, the activator/inhibitor model of trichome initiation and inhibition relies on the MBW tri-protein activation complex consisting of GL1 (MYB), GL3/EGL3 (bHLH) and TTG1 (WD40). This complex induces expression of trichome activators such as *GL2* and *TTG2* as well as inhibitors such as single-repeat R3MYBs within the same cell (**Fig. 1.3**; reviewed in Ishida *et al*., 2008). Single-repeat R3MYBs compete with R2R3MYB GL1 to bind with GL3 (Wang *et al*., 2010). GL1 acts locally within individual cells, whereas single-repeat R3MYBs such as TRY can act over a longer distance by diffusing via plasmodesmata, possibly resulting in an imbalance between GL1 and TRY in neighboring cells that will determine trichome/cell fate (reviewed in Ishida *et al*., 2008). Using microinjections of labelled TTG1 proteins, Bouyer *et al*., (2008) showed that TTG1 protein is moved actively to incipient trichome cells via plasmodesmata by increasing their size exclusion limit. TTG1 is then restrained by GL3 within trichomes (Balkunde *et al*., 2011). Over-expression (OE) of *GL1* in weak (moderately reduced transcript) *ttg1* Arabidopsis backgrounds results in trichome clusters, whereas in strong (strongly reduced transcript) *ttg1* backgrounds, *GL1* OE results in a glabrous phenotype (Larkin *et al*., 1994). These expression studies in mutant variants provide evidence that relative dosage of primary regulators is important in selecting the trichome/cell pathway (Larkin *et al*., 1994).

Over-expression of *TTG1* in an Arabidopsis wild type background does not result in increased trichome numbers (Payne *et al*., 2000) and the same is true for OE of *TTG1* in the *B. napus* cv Westar background. However, OE of *TTG1* has not been reported in an Arabidopsis *GL3* over-expression background. *BnTTG1* over-expressed in the AtGL3^{+} *B. napus* background resulted in a glabrous phenotype in leaves of O-3-7. This phenomenon did not result from co-

suppression of *BnTTG1* since O-3-7 showed increased *TTG1* expression in all three tissue sets tested. Instead, it suggests an inhibitory role for *TTG1* in the *B. napus* trichome development pathway. The sequence of *BnTTG1* coding region is 81% homologous to the Arabidopsis *TTG1*, but the species-specific and trichome-specific nature of different Brassica *TTG1* genes need to be examined to determine if the *B. napus TTG1* has particular inhibitor-specific sequence features. In addition, transformation with a functional (activating) *AtTTG1* gene and experiments to determine proteins and genes that *BnTTG1* interacts with are needed to confirm an inhibitory function for this gene. Moreover, the question of whether *B. napus* trichome initiation is similar or different to the Arabidopsis models needs to be examined.

In Arabidopsis, GL3 protein can bind to its own promoter to regulate its expression in a negative feedback mechanism (Morohashi *et al*., 2007). Over-expression of *AtGL3* transcripts in *B. napus* resulted in a lower expression of *BnGL3* in $AtGL3^{+}$ plants compared to Westar plants suggesting that the *B. napus GL3* promoter is responding similarly to the AtGL3 protein, although this will only be proven by a pair-wise comparison of transcription and protein accumulation levels for both *AtGL3* and *BnGL3*, as well as protein-promoter binding studies. Regardless, if the increased trichome phenotype in hairy K-5-8 is due to the high expression of *AtGL3*, high *AtGL3* expression observed in the glabrous O-3-7 line also has to be considered in order to understand the true role of *BnTTG1* and *GL3* in Brassica trichome development. These two opposite phenotypes can be reconciled by conceiving of the lifting of an inhibitory action of a proposed inhibitor BnTTG1 protein on GL3 protein in TTG1-reduced K-5-8 and that potentially high levels of AtGL3 protein are binding and depleting increased levels of BnTTG1 protein in O-3-7 such that insufficient GL3 remains in O-3-7 to bind with GL1 protein (**Fig. 2.32**). Furthermore, based on the proposed second mechanism for trichome initiation in Arabidopsis where TTG1 is depleted from neighbouring non-trichome cells by GL3 trapping in trichome cells (Bouyer *et al*., 2008; Balkunde *et al*., 2011), less TTG1 protein presumably available in the TTG1-down regulated K-5-8 cells will titrate less GL3 and leave more GL3 to associate with GL1 (**Fig. 2.32**). TTG1 may also be regulating GL3 through post-transcriptional or post-translational mechanisms, which are apparently not known for GL3 in Arabidopsis. In Arabidopsis, TTG1 is known to regulate another bHLH protein TT8 through a post-translational mechanism (Baudry *et al*., 2004).

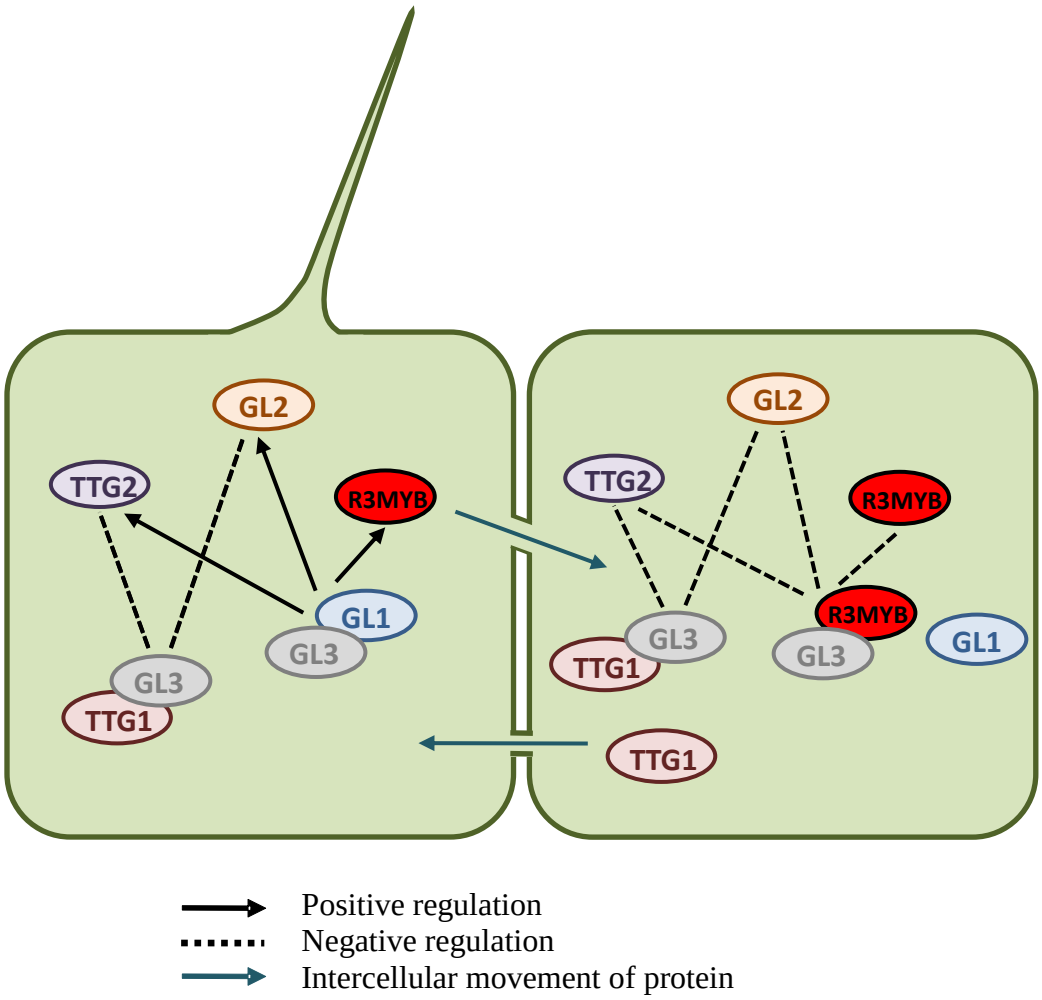


Fig. 2.32. Di-protein regulatory model for trichome initiation in *B. napus*. The GL1-GL3/EGL3 complex promotes *GL2*, *TTG2* and *R3MYB* expression. The TTG1-GL3/EGL3 complex negatively regulates the expression of *GL2* and *TTG2*. The R3MYB proteins move into neighbouring cells where they compete with GL1 for binding to GL3/EGL3. TTG1 potentially is an inhibitor of the di-protein complex that stimulates trichome initiation, but physical evidence is lacking. Neither the R3MYB-GL3/EGL3 nor the TTG1-GL3/EGL3 complexes or dissociated GL1 can promote *GL2*, *TTG2* or *R3MYB* expression. Cells expressing *GL2* and/or *TTG2* differentiate into trichome cells (reviewed in Ishida *et al*., 2008). R3MYB represent TRY, CPC, ETC1 or CPL3. Modified from Ishida *et al*., 2007; Wang *et al*., 2008; Wang *et al*., 2010; and Gan *et al*., 2011.

The increased trichome density phenotype of *B. napus* K-5-8 and the decrease in trichome density that restores a wild type phenotype to O-3-7 correlates very clearly in *B. napus* leaves with the expression pattern of *GL2*, although the expression of *BnGL2* in AtGL3^{+} *B. napus* leaves is confusing since it does not correlate with trichome density. Hence, factors, such as *GL2*, have to be considered for whether they function in a similar fashion as in Arabidopsis (**Fig. 2.32**). Over-expression of *GL1* alone was able to induce *GL2* expression in leaf mid vein and margin in both wild type Arabidopsis and a *ttg1*mutant, suggesting that *TTG1* is not an absolute requirement for GL2-dependent trichome induction (Szymanski *et al*., 1998). Moreover, co-transfection of GL1 and GL3 without TTG1 was also capable of activating endogenous *GL2* expression, but neither GL1 nor GL3 individually had any effect on GL2 expression (Wang and Chen, 2008). Co-transfection of GL3 and TTG1 was also not able to induce the expression of the *GL2* gene in Arbidopsis suggesting that TTG1 may not be required for activation of *GL2*, but rather for stability of the GL3/GL1 complex. Activation of *GL2* transcription requires direct binding of GL1 and GL3 to the promoter region of *GL2* (Wang and Chen, 2008). Excess presence of TTG1 may form an inactive TTG1-GL3 di-protein complex which can still bind to the *GL2* promoter masking the GL1-GL3 di-protein binding which can otherwise activate GL2 transcription (**Fig. 2.32**). ChIP experiments have shown that in Arabidopsis *GL2*, *CPC* and *ETC1* are direct targets of TTG1, suggesting that TTG1 and GL3 share many of the same targets (Zhao et al., 2008). Consequently, we must consider the possibility that in *B. napus*, the tri-protein complex is different or it regulates a different pathway of trichome initiation compared to Arabidopsis. A possible candidate to examine in the future for involvement in regulation of *B. napus* trichome development is *TTG2*, since its expression is regulated in Arabidopsis by TTG1 and GL3 (Zhao *et al*., 2008). TTG1 (Zhao et al., 2008), R2R3 MYB proteins and R3MYB proteins bind to the TTG2 promoter to regulate its expression (Ishida et al., 2007). TTG1 protein has been found both in the nucleus and the cytoplasm, so it could function both as a transcriptional co-regulator in the nucleus and as a protein-interacting factor in the cytoplasm (Zhao et al., 2008).

2.4.5. *TTG1* regulates *B. napus* anthocyanin similarly to regulation in Arabidopsis

In Arabidopsis, *bHLH* genes are involved in several TTG1-dependent pathways including trichome and root hair development, anthocyanin biosynthesis, production of seed coat mucilage and seed coat pigmentation (Balkunde *et al*., 2011). For example, *TT8* is required for

anthocyanin accumulation in leaves and seed mucilage biosynthesis, and *GL3* and *EGL3* have been identified as homologues to *TT8* (Baudry *et al*., 2006). These three bHLH proteins (GL3, EGL3 and TT8) all physically interact with TTG1 in yeast two hybrid assays (Balkunde *et al*., 2011), and a MBW tri-protein complex similar to the trichome MBW regulates anthocyanin accumulation (Baudry *et al*., 2004).

In this study on *B. napus*, the expression of the *bHLH* factor *AtGL3* resulted in increased anthocyanin accumulation in above-ground *B. napus* seedling tissues; however, the combination of *AtGL3* over-expression and knock-down of the WD40 factor, *TTG1*, resulted in lower anthocyanin levels than Westar. These two latter genes regulate anthocyanin accumulation in vegetative tissues of Arabidopsis by regulating late biosynthesis genes (reviewed in Petroni and Tonelli, 2011). In Arabidopsis, over-expression of *GL3* in a wild type background up-regulates expression of the structural genes *DFR* and *ANS* that are down-regulated in *ttg1* mutants (Gonzalez *et al*., 2008), and the promoters of both *DFR* and *ANS* are activated by the MBW complex (Feyissa *et al*., 2009). Similarly, expression of *AtGL3* in *B. napus* in this thesis research resulted in increased *DFR* and *ANS* transcript levels. However, when coupled with *TTG1* knock-down, *DFR* and *ANS* transcript levels decreased below Westar control levels. A previous study showed that over-expression of the Arabidopsis *MYB* gene *PAP1* in *B. napus* also increased anthocyanins and the expression levels of *DFR*, *ANS*, *F3H*, *F3'H*, and *GST* (Li *et al*., 2010), consistent with the response of *PAP1* over-expression in Arabidopsis (Tohge *et al*., 2005).

A MBW tri-protein complex has also been implemented in the production of seed coat mucilage by regulating the expression of *MUCILAGE-MODIFIED4* (*MUM4*) (Western *et al*., 2004). Reduced seed coat mucilage has been observed in Arabidopsis *ttg1*, *ttg2* and *gl2* mutants (Western *et al*., 2004). However, no seed mucilage was observed on any of the *B. napus* lines tested, probably due to the very low amount of seed mucilage naturally present in *B. napus* (Vose, 1974).

CHAPTER 2. THE FUNCTION OF THE *TRANSPARENT TESTA GLABRA1* GENE ON TRICHOME DEVELOPMENT, GROWTH, ANTHOCYANIN ACCUMULATION AND SEED MUCILAGE PRODUCTION IN *BRASSICA NAPUS* - Relationship to the thesis in its entirety

Chapter 2 relates to the thesis as it provides direct examples of how *BnTTG1* affects trichome patterning, growth and anthocyanin accumulation in the *B. napus* AtGL3^{+}background. Relatve expression of primary trichome and anthocyanin regulatory genes were affected due to altered expression levels of *BnTTG1*.

CHAPTER 3. FIELD EVALUATION OF GROWTH AND THE POTENTIAL OF HAIRY K-5-8 TO DETER CRUCIFER INSECT PESTS

In a CFIA approved confined field experiment, vegetative and reproductive growth of two hairy lines AtGL3^{+} and K-5-8 were tested against wild type *B. napus* cv Westar and *B. napus* cv Westar seeds treated with insecticide Helix XTra® seed treatment. The vegetative characteristics, percent emergence, plant height, area of 2nd, 4th and 6th leaves, and number of leaves per four week-old plant were compared. Hairy line K-5-8 showed vigorous growth similar to wild type *B. napus* cv Westar, with vegetative growth on a par with treated and untreated Westar except for the area of 2nd and 4th leaves. In contrast, AtGL3^{+} showed growth retardation during early stage vegetative growth. The reproductive parameters, number of branches of twelve week-old plants, number of pods per branch, number of seeds per pod, total seed weight and 100-seed weight were also measured. Similar to vegetative growth, K-5-8 showed a greater reproductive vigour compared to the other lines. In a laboratory bioassay, except on the stem and cotyledons, Diamondback moth (*Plutella xylostella* - DBM) adults laid more eggs on hairy leaves of K-5-8 than glabrous Westar. However, more feeding damage from young larvae was observed on Westar leaves than K-5-8 in both choice and no-choice feeding assays. In a field test of all four lines, the glabrous cotyledons of all four lines proved to be more resistant to Flea beetle (*Phyllotreta cruciferae* and *Phyllotreta striolata* - FB) feeding than leaves. The hairy K-5-8 showed between 30-50% reduction in feeding over four ratings on 28 day old seedlings. These data support the introduction of the trichome phenotype into glabrous *B. napus* to increase host plant resistance against crucifer insects such as diamond back moth and FBs.

3.1 Introduction

Plants have a variety of defences against numerous insect pests. These defences may be biochemical, morphological or both, and are aimed at deterring insects from feeding and ovipositing. Defences can be either constitutively expressed or induced upon an attack, localized to the site of attack or systemic (reviewed in Kessler and Baldwin, 2002). Host plant resistance is categorized into three groups: i) antibiosis, which results in increased mortality or reduced longevity and reproductive success of the insect; ii) antixenosis, which affects the behaviour of the insect and is often expressed as non-preference of the insect for a resistant plant compared to a susceptible plant; and iii) tolerance, which is the ability of a plant to withstand or recover from

insect damage and perform better than a susceptible plant grown under similar conditions (Painter, 1951). Other than the economic benefit, plant resistance also has environmental benefits, e.g., reduced application of pesticides will reduce the accumulation of these harmful chemicals in the environment. In the U.S.A. alone, the annual application of pesticides has been reduced by nearly 6 million tons largely as a result of the introduction of Bt varieties of cotton and corn (Smith, 1999).

Glabrous canola crops are susceptible to a number of specialist and generalist insect pests during the growing season. FBs (*Phyllotreta cruciferae* and *Phyllotreta striolata*) and DBM (*Plutella xylostella*) are Brassica specialist insects with FBs being the most economically important pest of canola in Canada (www.canolacouncil.org, 11/6/2012). Young stands of canola from emergence to the fourth leaf stage, are the most susceptible to FB feeding. A heavy infestation can result in severe damage to the cotyledons, first leaves, petioles and stems. If FBs attack the meristem, an entire crop can be destroyed, resulting in growers reseeding or leaving the field fallow over the growing season (www.canolacouncil.org, 11/6/2012). Heavy foliar feeding from emergence to fourth leaf stage can result in seedling mortality, delayed and reduced seedling growth, delayed maturity, lower seed yields, and decreased seed grade (Westdal and Romanow, 1972). Plants have to be protected from FB attack over the first two to three weeks following emergence to prevent this level of damage. The economic impact of FB feeding varies with their population density which in Canada is affected by changes in the weather. In spite of the 40 million dollars spent each year on insecticides in years of FBs abundance, up to 10% yield losses are common across the Canadian prairies (www.canolacouncil.org, 11/6/2012).

Next to FBs, DBM (*Plutella xylostella*) is one of the most destructive pests of Brassicaceae crops worldwide. DBM larvae feed on any exposed tissues of plant species in the mustard family, canola being a primary target in Canada. A considerable variation in the severity of DBM infestation from year to year and location to location can occur as a result of changing wind currents from southern Canada (www.canolacouncil.org, 11/6/2012). As a Brassicaceae specialist, *Plutella xylostella* is guided by host glucosinolates for host location, oviposition stimulation and feeding initiation (Sarfraz *et al*., 2011). The annual cost of trying to control DBM worldwide is approximately $1 billion (reviewed in Talekar and Shelton, 1993).

Currently, control of FB and DBM is almost entirely dependent on pesticide application, with more than 90% of canola seeded annually in North America treated with systemic insecticidal seed treatment for FB control (Soroka *et al*., 2008). Approximately 50-60 insecticide applications can be made every year to try and control DBM in certain Brassicaceae vegetable growing regions of the tropics (Amit *et al*., 2004). Due to this constant high selection pressure, both FB and DBM have developed resistance to a number of these insecticides (reviewed in Talekar and Shelton, 1993). As a result, development and adoption of insect-resistant canola cultivars would contribute to a reduction in costly and hazardous pesticide applications.

Trichomes (leaf hairs) have evolved as an effective physical defence mechanism against herbivore feeding and oviposition. Trichomes develop at the distal end of a developing leaf, with the youngest stages located at the proximal end, thereby protecting the more valuable emerging young region of the leaf (Woodman and Fernandes, 1991; Larkin *et al*., 1996). The effect of the presence/absence of trichomes on specialist insect pest feeding has been studied in several Brassica plant species including *B. rapa, B. napus* and *B. villosa* (Agren and Schemske, 1993; Palaniswamy and Bodnaryk, 1994). Trichome density and/or length both negatively impact feeding and oviposition of many insects. Mature leaves of *B. villosa* with a high density of trichomes are extremely resistant to FB damage compared to *Brassica spp* with low leaf trichome densities (Palaniswamy and Bodnaryk, 1994). Recently, Soroka *et al*. (2011) demonstrated an increased resistance to FB feeding compared to glabrous *B. napus* cv Westar in a transgenic *B. napus* cv Westar hairy line resulting from the expression of the *AtGL3* gene. The level of resistance was similar to or higher than that observed in cultivars grown from insecticide-treated Westar seeds. Oviposition and feeding damage by DBM was also somewhat decreased in the $AtGL3^{+}$ hairy canola seedlings compared to glabrous Westar lines (Adamson, 2008). The presence of trichomes has also been observed to reduce oviposition of DBM on Arabidopsis (Handley *et al*., 2005). An increased trichome density can result in behavioral changes in FBs where they simply walk away from leaf discs with a high density of trichomes (Palaniswamy and Bodnaryk, 1994). Thus, the development of new canola germplasm with increased trichome densities should increase host plant resistance to a number of specialist and non-specialist insect pests.

3.2 Material and methods

3.2.1 Plant material

For field evaluations of growth and FB feeding, untreated seeds of glabrous *B. napus* cv Westar, homozygous AtGL3^{+} hairy *B. napus* in a Westar background, homozygous hairy K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) and glabrous *B. napus* cv Westar seeds treated with the insecticide Helix XTra® (Syngenta Crop Protection Canada, Guelph, ON, Canada, consisting of 20.7% thiamethoxam, 1.24% difenoconazole, 0.39% metalaxyl-M, and 0.135% fludioxonyl) were used. Hairy K-5-8 and glabrous *B. napus* cv Westar were used to study the effect of trichomes on DBM oviposition and feeding.

3.2.2 Plants with novel trait field trial design and growth evaluation

The four *B. napus* entries were tested in a CFIA-approved confined field site at the Saskatoon Research Centre (AAFC) between May – August, 2011 with the assistance of the field crew of Dr. J. Soroka and Dr. M. Gruber. A randomized complete block design (RCBD) was used with four replicates. Each replicate consisted of four rows for each plant line, with 200 seeds per row. Percent seedling emergence in either middle row was counted up to 27 days after seeding in each replicate for each line. Plant height, number of leaves per plant, and leaf area were measured on five four-week-old plants per line per replicate. Leaves were harvested and immediately transferred to a container with ice and brought to the laboratory. Leaf area was measured using a digital image analysis system (Pseudo-colour Ag-Vision®, Decagon Device, Inc, Pullman, WA). To comply with reproductive containment, pollen-proof net cages of 1 m x 1 m were laid over a subset of plants in each replicate of each line at bolting and the netting edges sealed with soil. At the end of the trial when flowering was complete, the cages were removed and the number of reproductive branches was measured on three 12-week-old plants per line. The number of pods was counted on three randomly selected branches for each of these plants and seeds were counted from 10 pods for each of these plants. Seed yield per plant and 100-seed weight were compared between these plants. A germination test for field-harvested seeds was conducted in CoCo mix media (see **Appendix 6.4**) for 10 days under greenhouse conditions (22/18^{0}C temperature regime and a 16/8 h light/dark photoperiod (230 µE.m^{-2}.s^{-1})).

3.2.3 Flea beetle feeding bioassay

The CFIA-approved field trial was used to determine FB damage ratings for the hairy and glabrous *B. napus* lines. Transgenic hairy lines K-5-8 and AtGL3^{+} and non-transgenic glabrous Westar and Westar treated with Helix Xtra® insecticide were compared for FB feeding damage on cotyledons, and 1st, 2nd, 3rd and 4th leaves. Feeding damage was determined at four different intervals, 14, 18, 22 and 28 days after seeding, using a visual rating scale ranging from 1 to 10. Ten plants from the middle two rows of each replicate per line were selected for feeding damage rating. Feeding damage of 10 % was rated as 1 whereas 100 % was rated as 10. A rating of 0.1 was given for a small patch of feeding of less than 10 % of the leaf. The damage ratings were multiplied by 10 and averaged to estimate the percentage of area damaged.

3.2.4 Diamondback moth oviposition bioassay

Ten hairy K-5-8 and 10 glabrous *B. napus* cv Westar were germinated in plastic vials (**Fig. 3.1**) under insect-free and insecticide-free conditions inside a growth chamber with a 22/18^{0}C temperature regime and a 16/8 h photoperiod of 400 µE.m^{-2}.s^{-1}. The laboratory colony of *P. xyllostella* was maintained on potted *B. napus* plants at 22-26 ^{0}C and a 12/12 h light/dark photoperiod (50 µE.m^{-2}.s^{-1}) in a white-walled growth chamber. Eleven day-old seedlings were transferred to mesh cages (57 cm x 57 cm x 57 cm) and 10 female and 10 male DBM adults were introduced into each cage. Seedlings were watered daily by hand with a small spray bottle. Female moths were given access to male moths for at least 48 h to allow mating prior to the experiment. The experiment was conducted for seven days under 22-26 ^{0}C and a 12/12 h light/dark photoperiod (50 µE.m^{-2}.s^{-1}) in a white-walled insect rearing growth chamber (**Fig. 3.1**). Hairy K-5-8 was used as the test hairy line, whereas hairy AtGL3^{+} had been tested against *B. napus* cv Westar in a previous study (Adamson, 2008). The number of eggs laid was counted on six different tissues (stem, cotyledons, and 1st - 4th leaves), over three time periods with a two day interval between periods, using both a dissecting microscope and a handheld magnifying glass. Moths were provided with a 10:1 water:honey solution placed in a tall plastic container with a wick and mesh covering to prevent drowning. This experiment was replicated three times.

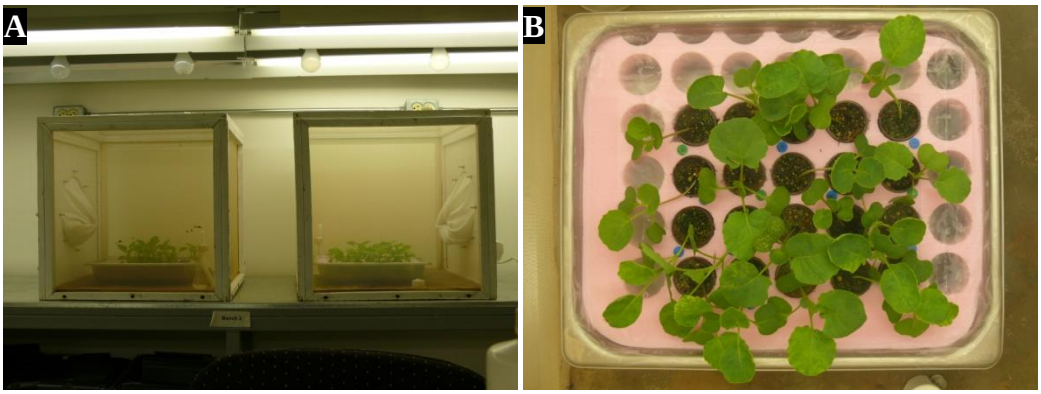


Fig. 3.1. Experimental setup for the DBM oviposition bioassay. A) Cages with trays containing ten 11-day-old seedlings each of *B. napus* cv Westar and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*), B) close top-down view of a tray containing randomly arranged test seedlings.

3.2.5 Diamondback moth feeding bioassay

To examine the effect of trichomes as a feeding deterrent on DBM larvae, choice and no-choice feeding bioassays were carried out between *B. napus* cv Westar and K-5-8. In the choice assay, eight 2nd-to-3rd instar diamondback larvae reared on potted *B. napus* plants at 22-26 ^{0}C and a 12/12 h light/dark photoperiod (50 µE.m^{-2}.s^{-1}) in a white-walled growth chamber were introduced into each of eight mesh cages (30 cm x 20 cm x 20 cm), each cage holding one 17-day-old Westar and one same age K-5-8 plant. Total remaining leaf area was measured on each of the 1st-to-8th leaves using a digital image analysis system (Pseudo-colour Ag-Vision®) two weeks after the introduction of the larvae (**Fig. 3.2**). Similarly, pre-eaten leaf area was measured by covering the damaged area using sand. In a no-choice assay, two plants per line were caged together, and two cages per line were tested for feeding damage. Eight 2nd-to-3rd instar diamondback larvae were introduced into each of the four mesh cages (**Fig. 3.2**), using a bridge formed between aleaf from each of the two plant. The experiments were conducted under 22-26 ^{0}C, 12/12 h light/dark photoperiod (50 µE.m^{-2}.s^{-1}) in a white-walled insect rearing chamber.

Fig. 3.2. DBM 2nd to early 3rd instar larvae feeding bioassay on 17-day-old plants of *B. napus* cv Westar and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). Experimental setup for choice feeding bioassay and no-choice feeding bioassay. The two plants per cage were the same or different depending on choice versus no-choice feeding bioassay.

3.2.6 Statistical analysis

Data were analyzed using one-way ANOVA using a MIXED model in SAS 9.2 (SAS Institute, 2008). Assumptions of ANOVA were tested using a Normality test and Levene's test. Means were compared using Tukey test in SAS 9.2 and the treatments were declared significant at $P \leq 0.05$.

3.3 Results

3.3.1 Field evaluation of agronomical characteristics

In previous greenhouse experiments (section 2.3.4.2), growth and vigour of the new hairy K-5-8 line were measured. However, growth characteristics may differ when plants are grown in the field under differing soil composition and variable weather conditions. To test whether vegetative and reproductive growth of K-5-8 was affected by the increased production of trichomes, four entries (*B. napus* cv Westar [W], *B. napus* cv Westar treated with insecticide

Helix XTra® seed treatment [Wtrd], AtGL3^{+} and K-5-8) were tested in a CFIA-approved confined field experiment carried out between May – August, 2011 (**Fig. 3.3**).

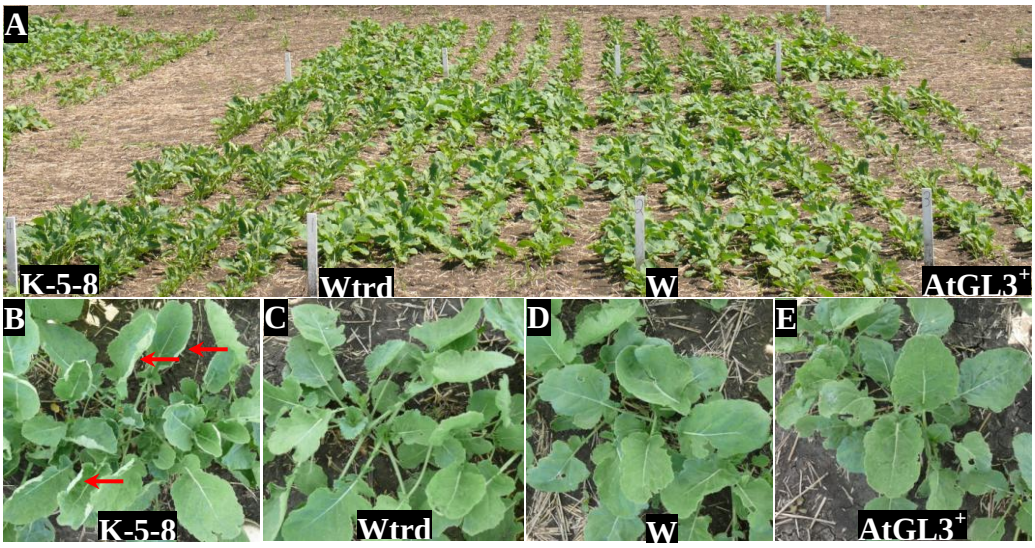


Fig. 3.3. Field evaluation of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A) View of growth of one entire replicate of four plant lines at 5 weeks after seeding. From left-to-right; four rows of K-5-8, Wtrd, W and AtGL3^{+}. B,C,D,E) Growth of individual plants at 5 weeks after seeding. B) Arrows show upward curling of leaves in K-5-8.

Seedling emergence: Emergence of seedlings was counted on five different days after seeding. Over the first three measurement periods at day 4, 6 and 10 after seeding, high variability occurred and obscured any differences between the three lines, although a numerical trend was observed, with AtGL3^{+} having the highest % emergence, followed by Westar, Westar-treated and K-5-8, respectively (**Fig. 3.4**). However, there is no significant difference ($P \leq 0.05$) in % emergence between any of the lines over the first 10 days. After 14 days, Westar-treated plants showed the highest percent emergence and this was significantly higher than K-5-8, which had the lowest percent emergence. By 27 days after seeding, Westar and Westar-treated showed

a significantly higher ($P \leq 0.05$) percent emergence compared to AtGL3$^+$, which had the lowest emergence at 55%. At this time point, there was no significant difference between K-5-8 and the three other lines.

Plant height: Four weeks after seeding, K-5-8 were the tallest plants, with a plant height of 11 cm, which was significantly different ($P \leq 0.05$) to AtGL3$^+$ plants which were the shortest at 8 cm (**Fig. 3.5A**). *B. napus* cv Westar had the second highest plant height, but its greater variability prevented separation of the means from any of the other lines.

Leaf area: The areas of the second, fourth and sixth leaves were measured using a digital image analysis system. Largest area for the second leaf was observed for Westar-treated and Westar plants, and both were significantly different ($P \leq 0.05$) from the two hairy lines AtGL3$^+$ and K-5-8, which were similar (**Fig. 3.5B**). A similar pattern was observed for the fourth leaf where Westar-treated plants had the largest leaf area followed by Westar, K-5-8 and AtGL3$^+$, but significant differences only occurred between the mean leaf area of Westar-treated and AtGL3$^+$ due to the high variability shown for most of the lines except AtGL3$^+$. By the fourth leaf, the leaf area of the most hairy line K-5-8 was intermediate among all four lines. The area of the sixth leaf was highest in Westar compared to the other three lines and treated Westar and K-5-8 were intermediate ($P \leq 0.05$) between Westar and AtGL3$^+$ plants, with the lowest area. Upward curling of K-5-8 leaves was observed under these field conditions (**Fig. 3.3B**).

Number of leaves: *B. napus* cv Westar and K-5-8 had the highest number of leaves at four weeks after seeding (**Fig. 3.5C**), with a lower number of leaves on Westar-treated and AtGL3$^+$ plants ($P \leq 0.05$).

Branches: The highest number of reproductive branches per plant was recorded for K-5-8, but this was not significantly different to that for Westar-treated plants (**Fig. 3.5C**). AtGL3$^+$ plants had a significantly lower number of branches compared to the three other entries. Number of branches on Westar plants was significantly higher than that of AtGL3$^+$ plants, but lower than that of K-5-8.

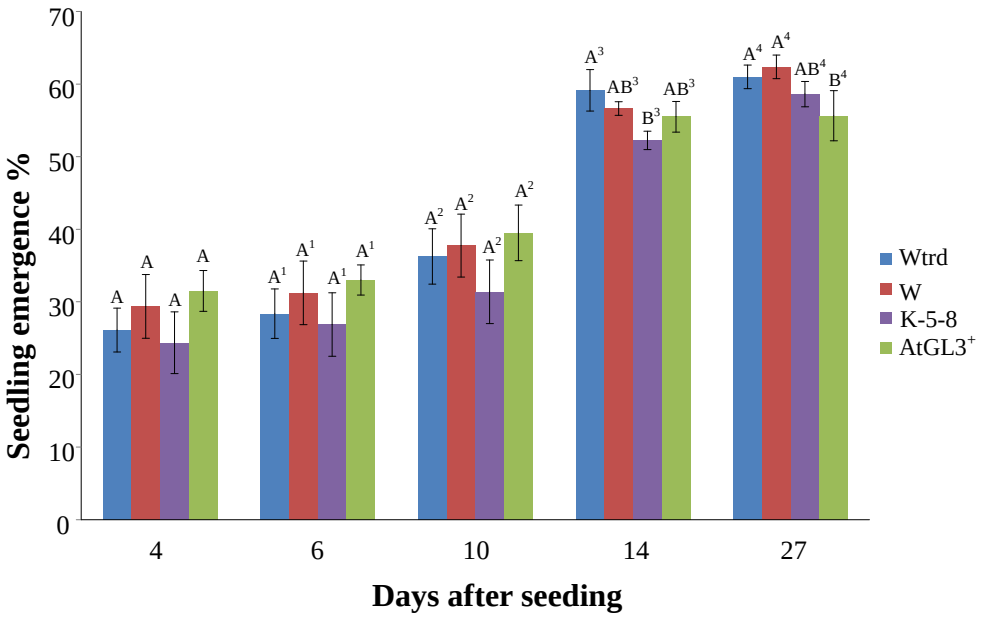


Fig. 3.4. Percent seedling emergence of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and line K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under field conditions using greenhouse grown seed. A Tukey test was carried out between the four plant lines at five different days after seeding. Means with the same letters do not differ significantly at $P \leq 0.05$, n=4 experimental blocks with all emerged plants counted within the two middle rows of four rows per plant line. The error bars show standard error of the means.

Pods and seeds: Several reproductive parameters were also tested for the four entries. Most number of pods per branch was recorded for K-5-8 followed by Westar-treated, Westar and AtGL3^{+} (**Fig. 3.5C**). However, due to high variability there was no significant difference ($P \leq 0.05$) between K-5-8, Westar-treated or Westar plants. The number of pods per branch in K-5-8 was significantly higher by more than 14 pods per branch than AtGL3^{+}, with lowest number at ~ 24 pods per branch. The number of seeds per pod followed a similar pattern with K-5-8 and Westar-treated having the highest number of seeds and AtGL3^{+} plants having significantly lower seeds per pod. Again, there was no significant difference ($P \leq 0.05$) between K-5-8, Westar-treated or Westar plants for this trait.

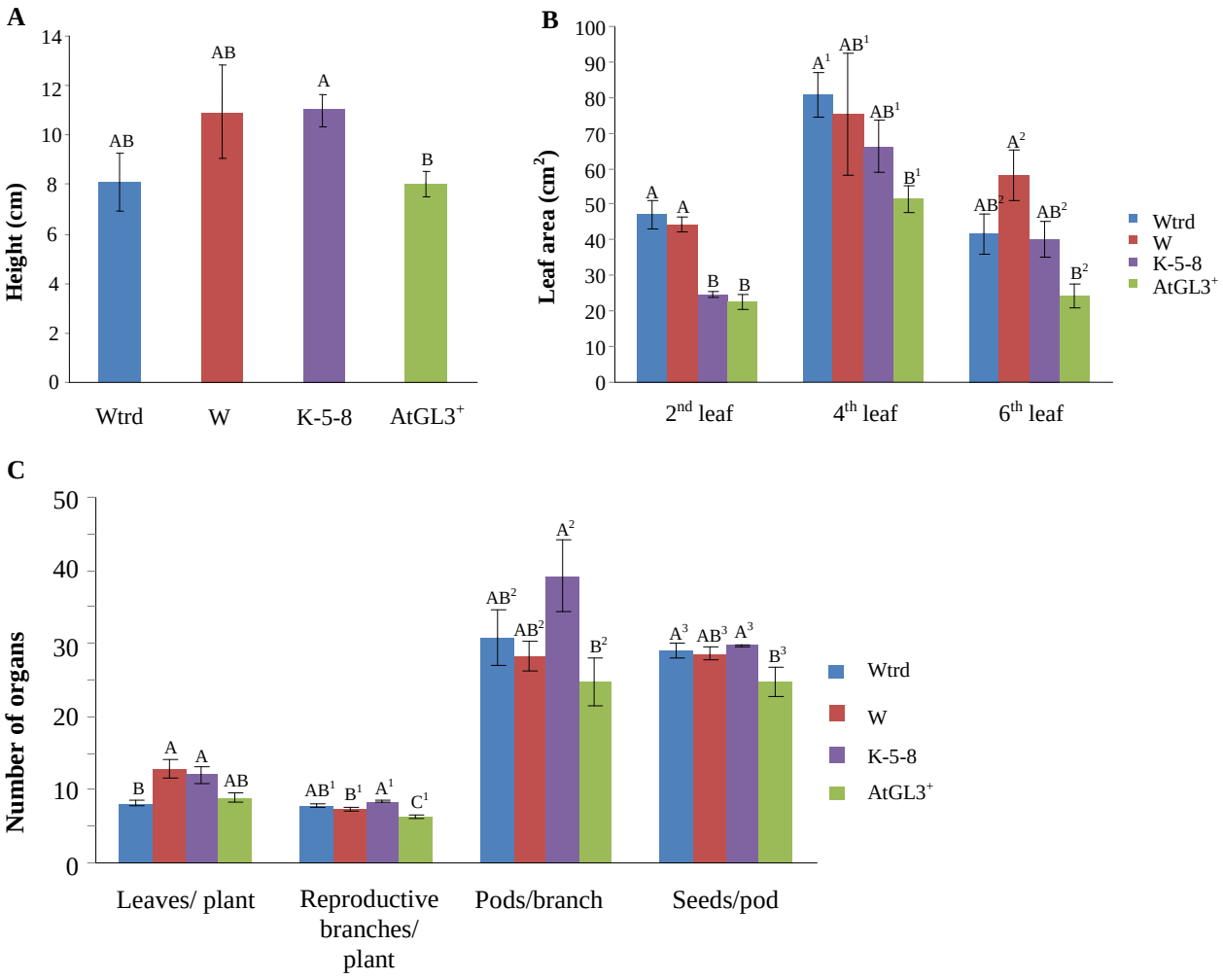


Fig. 3.5. Growth parameters of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*) under field conditions. A) Plant height, B) Leaf area and C) Vegetative and reproductive parameters. A Tukey test was carried out between the four plant lines for each growth parameter separately. Means with the same letter do not differ significantly at $P \leq 0.05$, n=4 experimental blocks. Plant heights and number of leaves/plant and leaf area were measured on five four-week-old plants per line per rep after seeding. Leaf area was measured using a digital image analysis system. Number of branches was measured on three 12-week-old plants per line per replicate after seeding. Number of pods was counted on three randomly selected branches for each of these plants. Seeds were counted on 10 pods for each of these plants. The error bars show standard error of the means.

Seed weight: The highest total numerical mean seed weight per plant was recorded for K-5-8 (**Fig. 3.6**), followed by Westar then Westar-treated and AtGL3^{+} plants. The mean numerical seed weight of K-5-8 was more than two-fold higher than the parental AtGL3^{+} line. However, due to high variability particularly within AtGL3^{+} and K-5-8 plants, significant differences ($P \leq 0.05$) between seed weights were not observed.

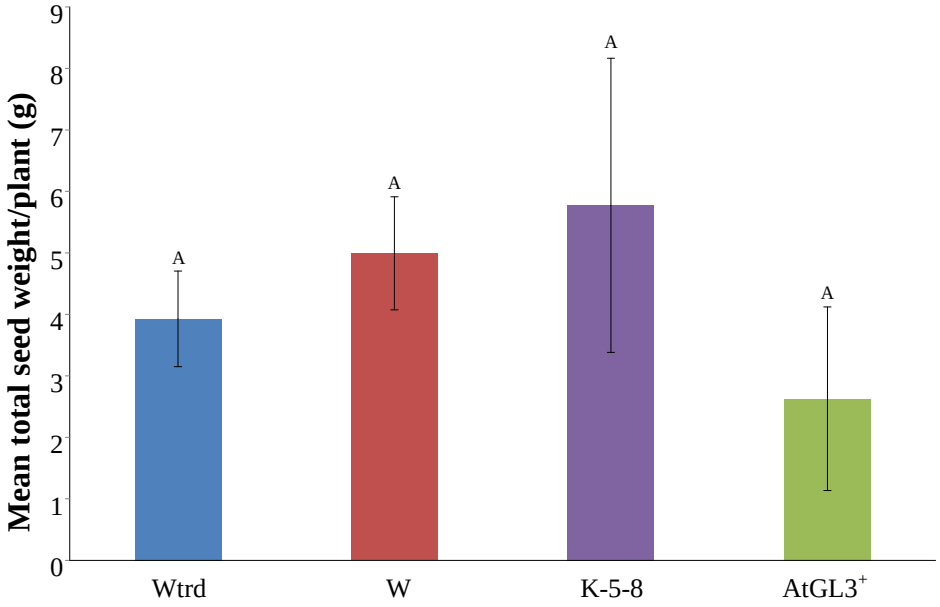


Fig. 3.6. Mean total seed weight (g) per plant of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and line K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under field conditions. A Tukey test was carried out between seed weights of each of the plant lines. Means with the same letters do not differ significantly at $P \leq 0.05$, n=4 experimental blocks. Seeds were collected and weighed from all plants within the 1 m x 1 m cage per line per replicate. The error bars show standard error of the means.

A similar pattern was also observed for the 100-seed weights (**Fig. 3.7**). K-5-8 and Westar plants showed the highest numerical 100-seed weight at 0.41 g. The lowest 100-seed

weight of 0.32 g was recorded for AtGL3^{+}. However, no significant difference between the means was determined for any of the lines tested, although the variability within lines was less than with total seed weight per plant (**Fig. 3.6**).

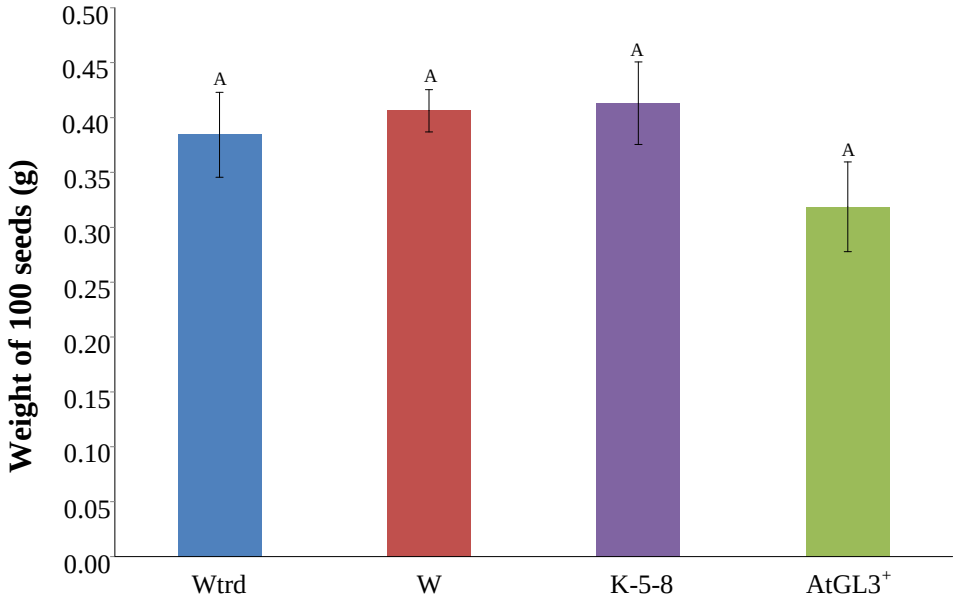


Fig. 3.7. Weight (g) of 100 seeds of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), homozygous AtGL3^{+} *B. napus* (AtGL3^{+}) and homozygous K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under greenhouse conditions. A Tukey test was carried out between seed weights of each plant line. Means with the same letter do not differ significantly at $P \leq 0.05$, n=4 experimental blocks. Seeds were bulked from all plants in a 1 m x 1 m cage per line per rep. Three technical replicates per line per replicate were weighed. The error bars show standard error of the means.

Seed viability: Viability of field harvested seeds was tested under greenhouse conditions. A germination test was carried out with 100 seeds harvested from each replicate of the four lines (**Fig. 3.8**). Similar to 100-seed weight, Westar and K-5-8 showed the highest numerical mean percent germination at over 90 %. The lowest percent germination (68.5%) was recorded for

AtGL3^{+} plants. However, no significant difference ($P \leq 0.05$) was detected between the means of any of the lines due to the high variation for both Westar-treated and AtGL3^{+} seeds.

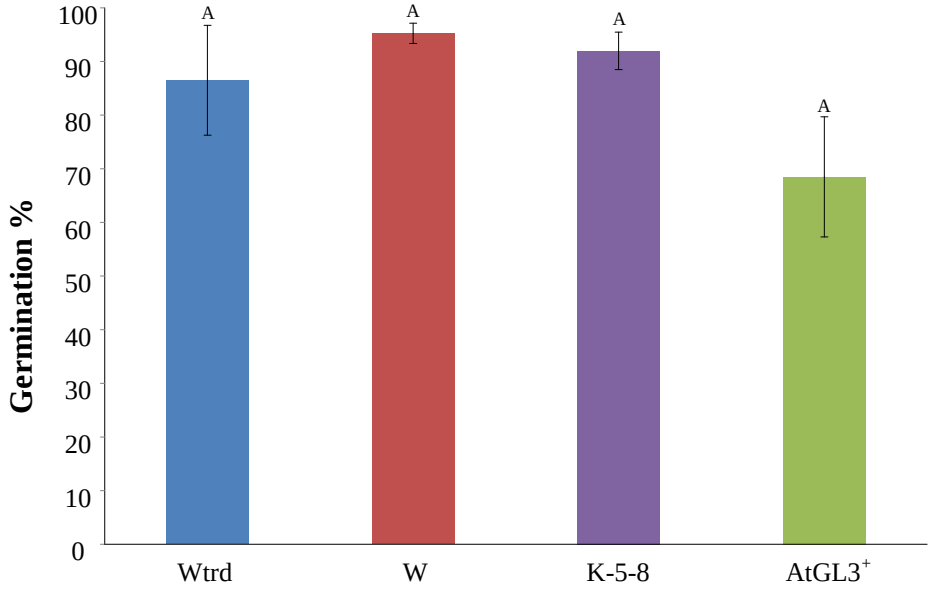


Fig. 3.8. Percent total germination of field harvested seeds of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and line K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). One hundred field harvested seeds bulked from all plants in a 1 m x 1 m cage per line per replicate were germinated under greenhouse conditions. A Tukey test was carried out between percent germination of each of the plant lines. Means with the same letter do not differ significantly at $P \leq 0.05$, n=4 experimental blocks. The error bars show standard error of the means.

3.3.2 Flea beetle field bioassay

In the field test, 14 days after seeding, Westar cotyledons had substantially higher feeding damage compared to the other three lines but the variability between plants was very high (**Fig. 3.9**). The significantly lowest ($P \leq 0.05$) level of feeding (less than 2% damaged area) was recorded for cotyledons of insecticide treated Westar. The mean damage sustained by K-5-8 and AtGL3^{+} cotyledons was very similar to that of Westar-treated cotyledons, but the high variability

of the untreated Westar cotyledons skewed the ANOVA analysis such that no significant difference ($P \leq 0.05$) was determined between K-5-8, AtGL3$^+$ and Westar. However, K-5-8 and AtGL3$^+$ both showed a strong trend towards significantly lower ($0.05 < P \leq 0.1$) FB feeding damage than Westar.

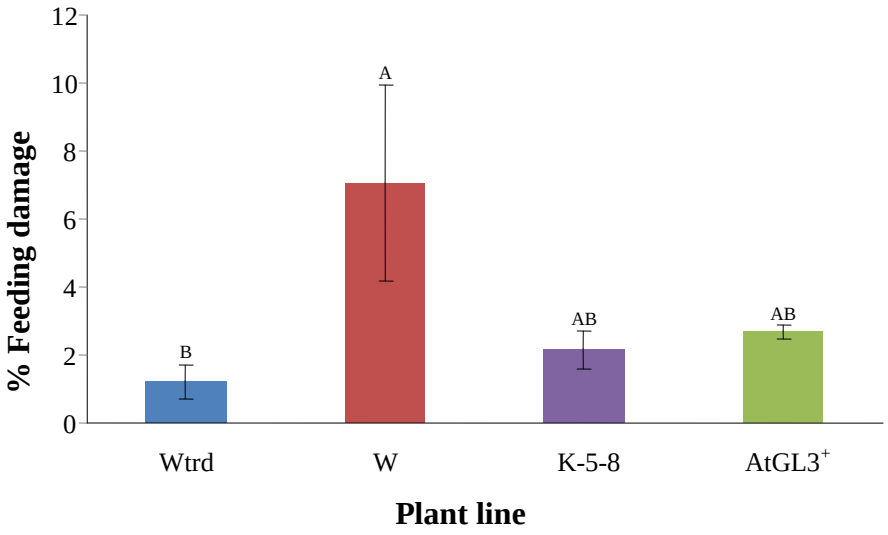


Fig. 3.9. FB feeding damage to the cotyledons of 14-day-old seedlings of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3$^+$ *B. napus* (AtGL3$^+$) and K-5-8 (*BnTTG1* knock-down in AtGL3$^+$ *B. napus*) under Saskatoon, SK field conditions. Percent feeding damage on the cotyledons of the *B. napus* seedlings at 14 DAS based on a visual rating scale. A Tukey test was carried out between the mean percent damaged area of the four plant lines. Means with the same letter do not differ significantly at $P \leq 0.05$, n=4 experimental blocks with 10 plants per line evaluated and averaged per block. The error bars show standard error of the means. DAS – days after seeding

A second rating was carried out on cotyledons and combined first and second leaves at 18 days after seeding. Similar to 14-day-old seedlings, Westar cotyledons were the most damaged and Westar-treated cotyledons the least damaged. Again, high variation in the level of damage on Westar cotyledons resulted in no significant difference between cotyledons of Westar-treated, K-

5-8 and AtGL3^{+}, although the damage inflicted on K-5-8 and AtGL3^{+} was much closer to that of Westar-treated plants' cotyledons than Westar cotyledons (**Fig. 3.10B**). Damage to the leaves of Westar was also three-fold higher ($P \leq 0.05$) than that to the leaves of the three other plant lines, with no significant difference between the leaves of K-5-8 and AtGL3^{+} or the Westar-treated line (**Fig. 3.10B**).

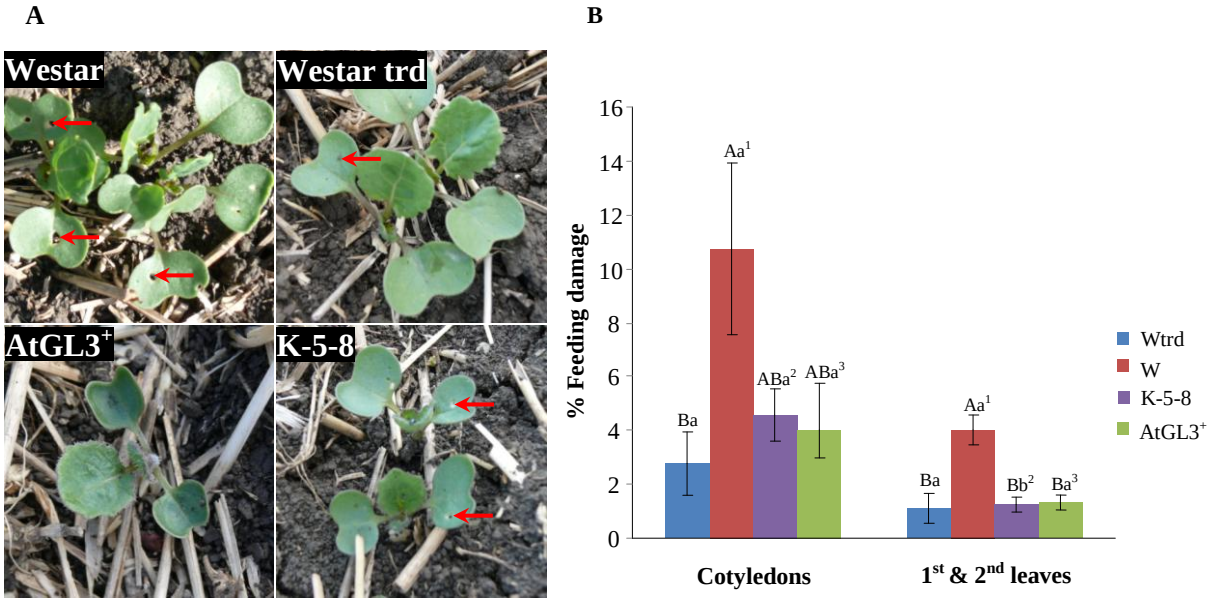


Fig. 3.10. FB feeding damage on 18-day-old seedlings of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under Saskatoon, SK field conditions. A) Typical FB feeding pits on the cotyledons of seedlings at 18 DAS. B) Percent feeding damage on cotyledons and first two leaves of the *B. napus* seedlings at 18 DAS based on a visual rating scale. A Tukey test was carried out to separate mean percent damaged area between the four plant lines for each tissue type (capital letters on bars). Within each line, a Tukey test was carried out between the means for the two tissue types (small letters on bars). Percent damage with the same letter do not differ significantly at $P \leq 0.05$, n=4 experimental blocks with 10 plants per line evaluated and averaged per block. The error bars show standard error of the means. DAS – days after seeding. Red arrows indicate damage.

In a cross-tissue analysis, mean damage to the leaves of Westar, Westar-treated and AtGL3^{+} seedlings did not differ statistically from that to their cotyledons, due to the high variability of damage on the cotyledons (**Fig. 3.10B**). However, leaves of K-5-8 sustained significantly lower damage ($P \leq 0.05$) compared to its glabrous cotyledons.

At 22 days after seeding, the trend for cotyledon damage was Westar (over 10%) > Westar-treated > AtGL3^{+} > K-5-8 (**Fig. 3.11**), however, there was no significant difference ($P \leq 0.05$) between each line. At 18 days after seeding, FBs appeared to have stopped feeding on

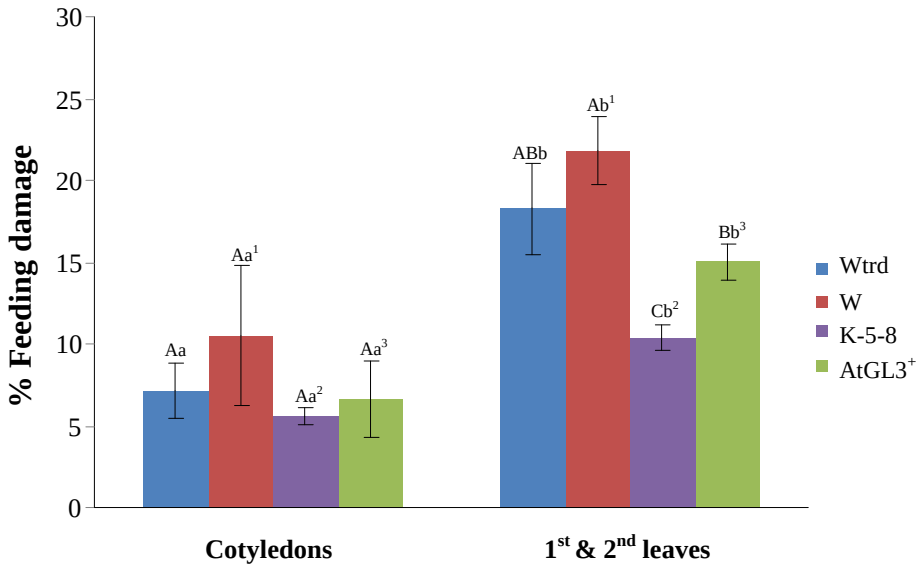


Fig. 3.11. FB feeding damage on 22-day-old seedlings of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under field conditions. Percent feeding damage was measured on the cotyledons and the combined first two leaves based on a visual rating scale. A Tukey test was carried out between the mean percent damaged area of the four plant lines for each of the two tissue types independently (capital letters on bars). Within each line, a Tukey test was carried out between the means for each tissue type (small letters on bars). Percent damaged with the same letter does not differ significantly at $P \leq 0.05$, n=4 experimental blocks with 10 plants per line evaluated and averaged per block. The error bars show standard error of the means.

Westar while continuing to feed on the other lines. By the 22 days after seeding stage of seedling development, leaves of all four lines sustained a significantly higher amount of damage than cotyledons. The highest amount of damage was still recorded on Westar, with the hairy leaves of K-5-8 showing the lowest amount of damage across all entries (2-fold lower than that of Westar). Both K-5-8 and AtGL3^{+} sustained significantly lower damage ($P \leq 0.05$) than Westar, with no significant difference between Westar-treated and AtGL3^{+}.

Damage levels on the cotyledons of the four plant lines were very similar between 22 days and 28 days after seeding (**Fig. 3.12**). AtGL3^{+} and K-5-8 plants showed the lowest amount of mean damage numerically, but again, high variation within several lines resulted in no significant difference between cotyledons for any of the lines. As in earlier developmental stages, the first two leaves of AtGL3^{+} and Westar plants at 28 days continued to sustain higher feeding damage compared to K-5-8, which had the lowest amount of damage of all lines ($P \leq 0.05$). In addition, for the first time in the trial, the first two leaves of AtGL3^{+} plants sustained higher feeding damage than Westar-treated plants. Westar plants had the highest feeding damage of all entries on 3rd – 4th leaves. This damage was significantly higher than that of K-5-8 and Westar-treated plants ($P \leq 0.05$).

As at 22 days, the feeding damage on leaves for all lines was higher at 28 days compared to that on their cotyledons ($P \leq 0.05$). The damage on both first two leaves and third and fourth leaves was over two-fold higher than that on the cotyledons. However, there was no significant difference in mean damage on both sets of leaves between any of the lines.

3.3.3 Diamondback moth lab bioassay

3.3.3.1 Oviposition bioassay

To study the oviposition preference of DBM adult females on hairy K-5-8 plants and glabrous Westar *B. napus*, choice oviposition bioassays were conducted in mesh cages in an insect rearing chamber. No significant difference ($P \leq 0.05$) was observed for the number of eggs oviposited on the stem and cotyledons between Westar and K-5-8, although egg laying on cotyledons showed substantially greater variation for both lines than on all other tissues (**Fig.3.13**). Second and third leaves of K-5-8 had significantly more eggs than those of Westar, while the higher number of eggs on the first and fourth leaves of K-5-8 did not differ significantly compared to Westar.

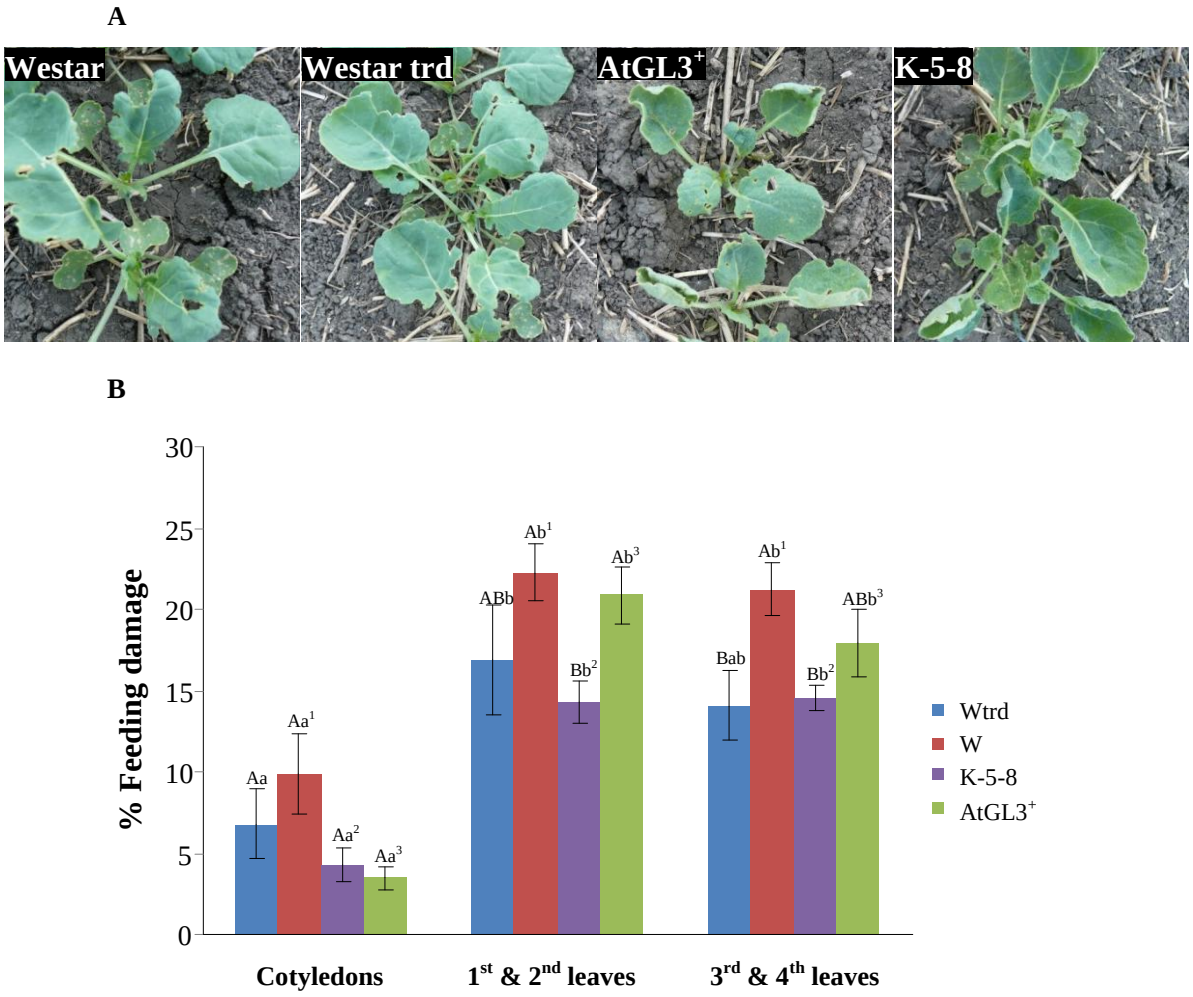


Fig. 3.12. FB feeding damage on 28-day-old seedlings of *B. napus* cv Westar (W), *B. napus* cv Westar treated with Helix XTra® seed treatment (Wtrd), AtGL3^{+} *B. napus* (AtGL3^{+}) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*) under field conditions. A) Typical FB feeding pits on the leaves of all four lines at 25 DAS. AtGL3^{+} plants were typically smaller than the other lines. B) Percent feeding damage on cotyledons, first two leaves (combined) and 3rd and 4th leaves (combined) at 28 DAS based on a visual rating scale. A Tukey test was carried out between the mean percent damaged area of four plant lines for each individual tissue type (capital letters on bars). Within each line, a Tukey test was also carried out between the means for each tissue type (small letters on bars). Percent damage with the same letter did not differ per block. The error bars show standard error of the means. DAS – days after seeding.

In glabrous Westar, the number of eggs laid on cotyledons (**Fig. 3.13**) was substantially higher ($P \leq 0.05$) than on any other tissue. While the high variability in egg counts resulted in no significant difference between the other tissues, a trend of stem > 1st leaf > 2nd leaf > 3rd leaf > 4th leaf was observed. The highest number of eggs was also found on the glabrous cotyledons of K-5-8 compared to the other tissues. As with Westar, the lowest number of eggs was laid on the 4th

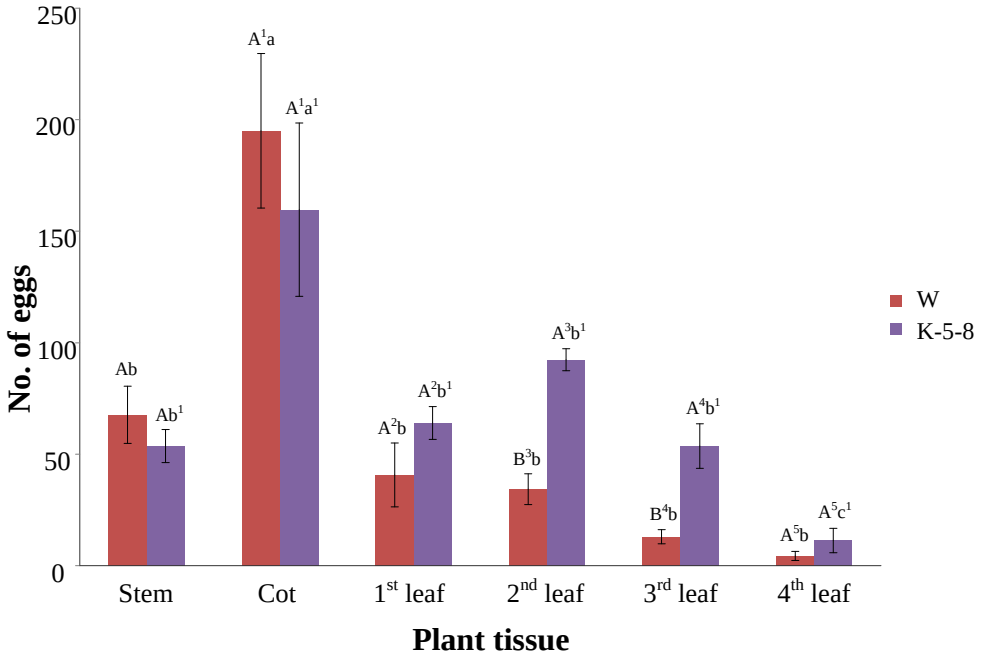


Fig. 3.13. Number of DBM eggs laid on different plant tissues of *B. napus* cv Westar (W) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). Numbers of eggs were counted on all tissues of all plants. A Tukey test was carried out to determine mean differences between the two plant lines for individual plant tissues (capital letters on bars only compare lines within a specific tissue). A 2nd Tukey test was carried out to test significantly different means between different tissues within a line (small letters on bars). Means with the same letters do not differ significantly at $P \leq 0.05$; n=3 experimental cages, each containing 10 plants per line. The error bars show standard error of the means. Cot – cotyledons

leaf of K-5-8 and this mean was significantly lower ($P \leq 0.05$) than those for the other leaves, stem and cotyledons. There was no significant difference between the number of eggs oviposited on the stem, 1st leaf, 2nd leaf and 3rd leaf of K-5-8 tissues and no obvious trend either (**Fig. 3.13**).

3.3.3.2 Feeding bioassay

3.3.3.2.1 Choice feeding assay

Eight 2nd-to-3rd instar DBM larvae were introduced into each of eight mesh cages, each cage holding one 17-day-old Westar and one K-5-8 plant. In six of the eight cages, glabrous Westar had more feeding damage than hairy K-5-8 (**Fig. 3.14A**). In cage seven, DBM larvae inflicted more damage on K-5-8 and in cage four, the damage was equivalent. Total mean feeding damage across all cages was significantly higher for Westar compared to K-5-8 ($P \leq 0.05$) (**Fig. 3.14B**).

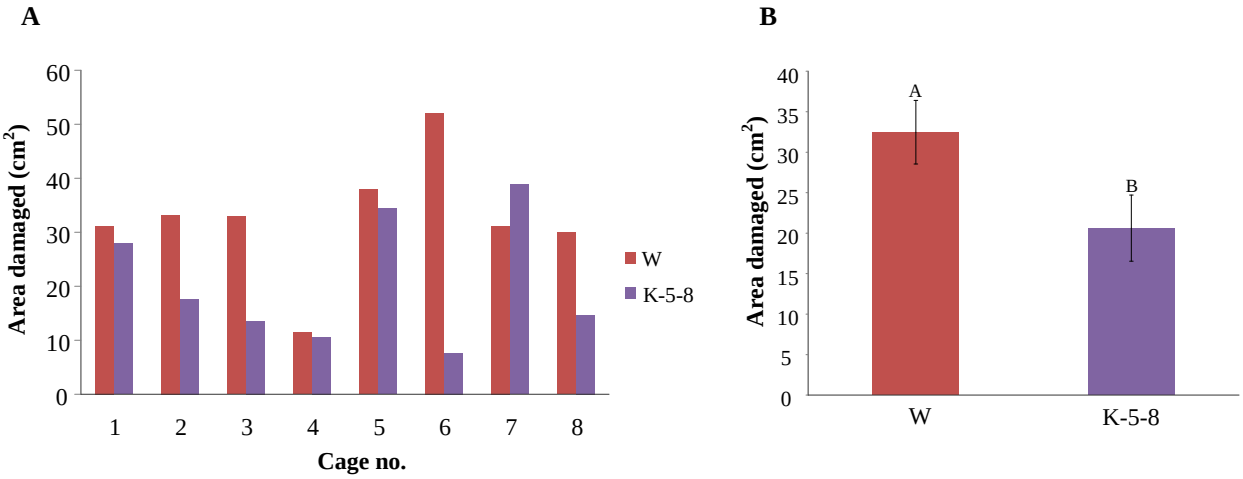


Fig. 3.14. DBM second to early third instar larvae choice feeding bioassay on 17-day-old plants of *B. napus* cv Westar (W) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). A) Total damaged leaf area (cm^2) for each plant in cages 1-8. B) Mean damaged leaf area (cm^2) per plant line across all cages for each line. Damaged Area was measured using a digital image analysis system. A Tukey test was carried out between the mean damaged leaf areas collectively for each of the two plant lines. Means with same letters do not differ significantly at $P \leq 0.05$, n=8 experimental cages each containing one plant per line. The error bars show standard error of the means.

3.3.3.2.2 No-choice assay

In a no-choice bioassay, two plants per line were caged together, and two cages per line were tested for feeding damage. 2nd-to-3rd instar larvae preferred feeding on glabrous Westar leaves than hairy K-5-8 leaves when given no choice. However, very high variability in feeding on the K-5-8 line resulted in no significant difference ($P \leq 0.05$) between the mean feeding damage on Westar and K-5-8 after two weeks of feeding (**Fig. 3.15**).

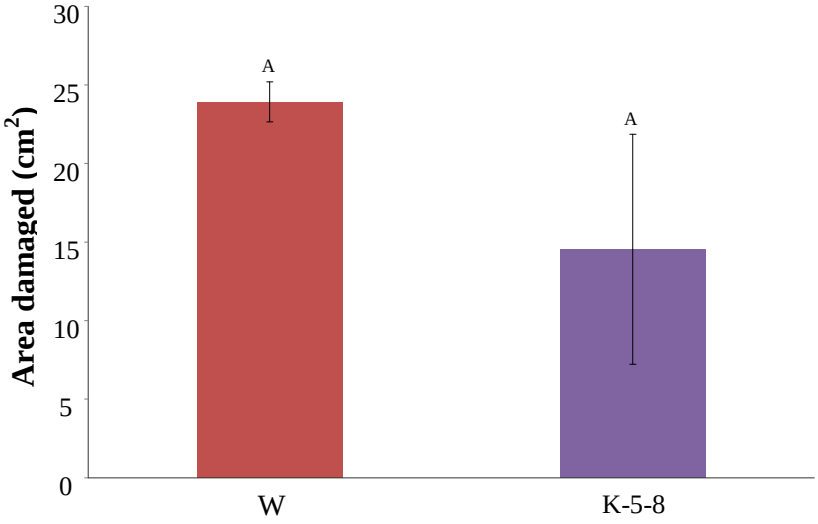


Fig. 3.15. DBM 2nd-to-3rd instar larvae no-choice feeding bioassay on leaves of *B. napus* cv Westar (W) and K-5-8 (*BnTTG1* knock-down in AtGL3^{+} *B. napus*). Mean damaged leaf area (cm^2) per plant line was measured using a digital image analysis system. A Tukey test was carried out between the mean damaged leaf areas collectively for each of the two plant lines. Means with the same letter do not differ significantly at $P \leq 0.05$, n=4 biological replicates. The error bars show standard error of the means.

3.4. Discussion

A very hairy transgenic *B. napus* line, resulting from the over-expression of the *AtGL3* gene and the under-expression of the *BnTTG1* gene (K-5-8), was tested under field conditions for germination, vegetative growth and reproductive success. The potential resistance of this new line against crucifer insects was also tested in a lab bioassay for DBM oviposition and feeding and in a CFIA-approved field bioassay for FB feeding.

With the introduction of K-TTG1 into the already hairy AtGL3$^+$ line, two lines, K-5-8 and K-6-3, that are agronomically competitive with wild type *B. napus* were produced and showed increased resistance to crucifer insects. With a slower germination rate than Westar control plants, K-5-8 may in fact produce more robust, stronger seedlings, which are less susceptible to insect pests. After germination K-5-8 plants were, for most agronomic traits, equal to or better than AtGL3$^+$ or Westar-treated plants. However, K-5-8 plants produced more trichomes on more leaves, resulting in very hairy plants. A higher density of trichomes will alter the leaf boundary layer, thus increasing gas exchange between the leaf and its surroundings (reviewed in Johnson, 1975). The resulting decreased resistance to CO_2 may result in an increased CO_2 availability that could lead to a higher photosynthetic rate. An increase in growth accompanied by trichome density changes similar to K-5-8 has been previously reported in *Populus alba* where over-expression of *MYB186*, a close homologue of *AtMYB106*, increased leaf trichome density, growth (by 35%) and photosynthetic rate (by 200%) (Plett *et al*., 2010). However, whole-transcriptome analysis found no increase in any poplar genes associated with increased growth such as cell wall synthesis or cell cycling genes (Plett *et al*., 2010).

Increase in the length and the density of trichomes did not affect the reproductive vigour of K-5-8, indicating that the allocation of resources to *B. napus* trichome production did not limit other physiological processes. It had been earlier argued that trichomes can be produced at a low or negligible cost in *B. rapa*, such that increased trichome production would have no effect on the number of flowers produced (Agren and Schemske, 1993), although later in this work, increased trichome production was associated with a delay in flowering (Agren and Schemske, 1993). However, a strong negative correlation occurred between early flowering start date and total flower number, and the significantly more pods observed on *B. rapa* plants with high

trichome densities was suggested to be due to the advantage of late flowering (Agren and Schemske, 1994).

Numerous studies have found that trichomes have a negative impact on feeding and oviposition responses of many insects. Density (and length) of trichomes can negatively impact the ability of Phyllotreta beetles to gain access to food, shelter, and oviposition surface (Soroka *et al*., 2011). A negative relationship between number of trichomes and oviposition preference of DBM on six naturally occurring populations of *Arabidopsis thaliana* has been recorded (Schillinger and Gallun, 1968; Handley *et al*., 2005). In a similar fashion, glabrous cotyledons of *B. napus* K-5-8 had the highest number of DBM eggs compared to other tissues. However, although the glabrous stem and cotyledons of Westar were preferred over K-5-8 tissues by DBM females for oviposition, the most hairy leaves of K-5-8 were preferred for DBM oviposition over the same glabrous leaves of Westar. In another study comparing glabrous Westar and hairy $AtGL3^{+}$, DBM showed no preference for hairy leaves. However, there was an inconsistency in this latter study, in that more eggs were oviposited on hairy $AtGL3^{+}$ leaves in one out of the three trials carried out (Adamson, 2008). In contrast to this thesis, that latter study also found more eggs laid on the leaves of both hairy and non-hairy lines rather than on the cotyledons (Adamson, 2008).

These contrasting results can be explained in two ways. First, although several studies have found a negative relationship between number of trichomes and oviposition preferences, DBM may in fact not be very selective in their oviposition preference with respect to trichome density. Sarfraz *et al*. (2011) showed that for oviposition, DBM actually preferred host plant species on which the fitness of their offspring was the greatest. Both this thesis research and one of Adamson's (2008) trials suggest that oviposition preference is not linked to larval performance, since more eggs were found on the hairy leaves of K-5-8 that suffered less larval feeding damage, than on the glabrous Westar leaves that suffered the most damage.

Secondly, it could be biochemical differences among the host plants that determine oviposition preferences. Host selection by specialist insect pests can be dictated by the chemical complexity of a plant, either by the presence of stimulants or the lack of deterrents (reviewed in Simmonds, 2001). DBM relies on the presence of a select array of glucosinolates for host location, oviposition stimulation and feeding initiation (Sarfraz *et al*., 2011). A role for

flavonoids in host recognition and acceptance by many adult insects has also been reported (Simmonds, 2001). Anthocyanins, which can be feeding deterrents at high concentrations but stimulants at lower concentrations for some insects (Simmonds, 2001) are also oviposition deterrents for the specialist ninebark beetle, *Calligrapha spiraeae* (Tenczar and Krischik, 2007). Other than for anthocyanins, a chemical analysis of the hairy *B. napus* lines was not carried out in this thesis. However, above-ground tissues of AtGL3^{+} seedlings did have significantly higher anthocyanin content compared to Westar tissues, while K-5-8 had the lowest anthocyanin content. The higher anthocyanin content of AtGL3^{+} did correlate with a higher resistance to DBM oviposition (Adamson, 2008). Similarly, more eggs were laid on K-5-8 which showed the lowest anthocyanin content of all three lines. However, a tissue specific comparison was not carried out for anthocyanin levels for any of these plants, and full chemical screens including glucosinolates and confirming feeding tests with purchased compounds need to be done to determine whether chemical composition plays a role in DBM interaction with the new hairy lines.

A role for trichomes as physical feeding deterrent against insects has been reported in numerous studies (Agren and Schemske, 1993; Palaniswamy and Bodnaryk, 1994; Soroka *et al*., 2011). Furthermore, non-glandular trichomes are mainly nutritionally poor structures due to high cellulose and lignin contents, and as such their presence negatively impact larval development (reviewed in Levin, 1973). In this study, non-glandular trichomes proved to be a source of resistance for *B. napus* leaves against DBM larvae feeding. Previously, a no-choice feeding assay, demonstrated that DBM larvae will inflict more feeding damage on Westar leaves compared to the hairy leaves of *B. napus* AtGL3^{+}, and a delay in initial feeding and slower movement across the leaf was observed on hairy leaves compared to the non-hairy leaves (Adamson, 2008). A slower movement across the leaf will make these larvae more vulnerable to predation, giving a secondary advantage to the host plant. Failure to find adequate and appropriate food during early larval stages of cereal leaf beetle (on pubescent wheat) also leads to slower development, increased mortality and smaller weights as well as to the increased likelihood of predation (Schillinger and Gallun, 1968). Since an increased resistance to DBM larvae feeding was observed in K-5-8 leaves, with the lowest level of anthocyanin, this increased leaf resistance is more likely a physical resistance due to the increased number of trichomes.

Field evaluation of these hairy *B. napus* lines in the presence of DBMs is now essential to determine the true level of resistance when other natural biotic and abiotic factors are present.

One of the most interesting and expected outcomes of manipulating trichome regulatory genes in *B. napus* has been the impact of those genes on cotyledon feeding by crucifer FBs. In an earlier study on FB feeding on seedlings, the presence of trichomes on AtGL3^{+} *B. napus* leaves reduced the ability of FBs to feed normally or to reach the feeding surface (Soroka *et al*., 2011). This was the case with the glabrous AtGL3^{+} *B. napus* cotyledons and for the K-5-8 cotyledons of this thesis study. In both studies, Westar cotyledons were still preferred by FBs, even though the cotyledons, the cotyledon petioles and the stem below the cotyledons of all four lines in these two studies were glabrous. Although the cotyledons became horizontally oriented later on as the plants grew, cotyledons on the hairy lines were vertically oriented at the time of emergence. This initial vertical orientation coupled with an upward curling of the cotyledons of hairy lines could contribute to the reduced preference by FBs. However, a modified chemical composition in these transgenic cotyledons due to the impact of the transgenes on other pathways cannot be ruled out. High levels of anthocyanins were recorded in the above-ground seedling tissues of AtGL3^{+} plants grown under increased light intensity and duration; however, since the anthocyanin content in similar tissues of K-5-8 were lower than those for Westar tissues, changes in the anthocyanin content cannot be the only effector for the increased resistance of K-5-8 cotyledons.

The preference by FBs for the glabrous leaves of Westar over the hairy leaves of *B. napus* AtGL3^{+} and K-5-8 lines suggests that the presence of trichomes does negatively impact FB feeding. Under cool conditions, FBs move into canola fields less rapidly than under warmer conditions, approaching seedlings by walking or hopping. In warmer temperatures, the FBs migrate usually by flight, landing on cotyledons and leaves (Soroka *et al*., 2011). The cooler temperatures that prevailed specifically during the second damage rating on June 6th (13.8^{0}C) may have been influential in FBs approaching seedlings by walking or hopping. The increased presence of trichomes on the petioles and leaf abaxial surfaces of AtGL3^{+} and K-5-8 plants prevented FBs from easily reaching the feeding surfaces of these plants, thereby reducing feeding damage to these two lines. However, with the warmer temperatures that occurred during the third and fourth ratings, FBs were able to fly and land on feeding surfaces, bypassing the petioles and abaxial surfaces. Under these conditions, increased trichomes together with longer trichomes on the adaxial surface of K-5-8 plants further reduced feeding damage over that of

AtGL3^{+} leaves. However, even the increased trichome densities of AtGL3^{+} and K-5-8 plants did not completely deter FB feeding, although the deterrence was usually at the level or better than pesticide-treated plants. A complete deterrence will probably be the result of a combined physical and chemical approach to combating FB feeding.

The presence of trichomes can improve plant resistance to insect pests indirectly by improving symbiosis with predatory insects. While a dense cover of trichomes can lower natural enemy foraging efficiency (reviewed in Price *et al*., 1980; Van Lenteren *et al*., 1995) and increase cannibalism (Simmons and Gurr, 2004), it can also promote arthropod-plant mutualism (Treacy *et al*., 1987; Messina *et al*., 1995; Morais-Filho and Romero, 2010). There are very few cases where insects can overcome strong trichome defenses; however, some specialist herbivores such as *Heliconius charithonia* are able to free themselves from entrapment by trichomes by eating trichome tips or by laying silk mats on trichomes (Cardosa, 2008).

During the process of crop domestication, many potent resistant traits of wild progenitors have been lost, resulting in an increased susceptibility of current domesticated crops to insect pests and pathogens. A reintroduction of these traits through conventional breeding programs or molecular approaches is essential for the redevelopment of resistance to insect pests and pathogens, especially in light of the increasing number of pests and pathogens developing resistance to the current synthetic chemicals. In this thesis, the development of insect resistance by introducing a trait (non-glandular trichomes) present on many wild Brassica has now been clearly demonstrated, moreover, a gene combination (expression of the *AtGL3* and down-regulation of the *BnTTG1*) that continues this resistance trait even in glabrous cotyledons while improving growth qualities has also been demonstrated.

CHAPTER 3. FIELD EVALUATION OF GROWTH AND THE POTENTIAL OF HAIRY K-5-8 TO DETER CRUCIFER INSECT PESTS - Relationship to the thesis in its entirety

Chapter 3 relates to the thesis as it describes how the presence of trichomes affects both plant vegetative and reproductive growth as well as resistance to crucifer insects. In the previous chapter growth and vigour of the new hairy line K-5-8, under greenhouse condition, were described. However, growth characteristics and resistance to insect feeding may differ when plants are grown in the field under variable soil composition and weather conditions.

CHAPTER 4. GENERAL DISCUSSION

Numerous studies have found that the density and length of trichomes negatively impact feeding and oviposition responses of many insects by hindering their ability to gain access to the plant surface (Schillinger and Gallun, 1968; Handley *et al*., 2005; Soroka *et al*., 2011). Findings that trichomes on *B. rapa* can be produced at a low or negligible cost (Agren and Schemske, 1994) suggest that this phenotypic trait can be exploited for germplasm enhancement and plant breeding programs in the Brassicaceae. Previous transformation of glabrous *B. napus* cv Westar with the Arabidopsis *GL3* gene resulted in increased trichome densities on early seedling tissues, however, this was coupled with a severe growth penalty (Gruber *et al*., 2006). The restoration of growth by the down regulation of the *BnTTG1* gene in the AtGL3^{+} *B. napus* genetic background and the maintenance of FB resistance also in the glabrous cotyledons and DBM and FB feeding resistance in trichome-bearing leaves has exciting implications for canola plant breeding programs to enhance host plant resistance to a number of crucifer insect pests, including DBM and FB. This would have a strong impact on farm income and reduce insecticide application world-wide, and especially in Canada. Canadian farmers lose approximately $200 million annually from FB damage alone despite $40 million spent annually on pesticide applications. Moreover, the trend towards increased seed yield in the *B. napus* K-5-8 line coupled with its increased insect pest resistance could provide useful breeding material for the canola industry.

The effect of the *TTG1* trichome regulatory genes at restoring plant growth has rarely (only once) been reported in the literature (Szymanski *et al*., 1998) and not in combination with the *AtGL3* gene as demonstrated here in *B. napus*. Arabidopsis plants over-expressing *GL1* together with an inducible form of the maize *bHLH R* gene, were only viable in the absence of the inducer when this transgene combination was present in a homozygous *ttg1* background, but the same combination was not viable in a wild type background (Szymanski *et al*., 1998). Over-expression of *GL3*, a functional homologue of the *R* gene, in both a wild-type *B. napus* background and an Arabidopsis *try* mutant background also resulted in a depressed growth phenotype indicating that *bHLH* genes can have a negative effect on plant growth (Gruber *et al*., 2006; Schnittger *et al*., 1999). Similarly, the depressed growth phenotype of *B. napus* AtGL3^{+} was restored to a wild type growth phenotype when *TTG1* was knocked-down in the K-5-8 line, but depressed again when *TTG1* was over-expressed in the *AtGL3*-enhanced background. Although, the manipulation of *BnTTG1* transcript levels alone (either up or down) in a *B. napus*

cv Westar background resulted in no changes to growth, collectively, these experiments suggest that the *TTG1* gene does acts together with *bHLH* genes to control plant growth and viability in *B. napus*.

The role of *TTG1* in Arabidopsis trichome development has been extensively studied in trichome initiation. *Ttg1* null mutants initiate very few trichomes (Larkin *et al*., 1994) whereas, *TTG1* over-expression in a wild type Arabidopsis background does not result in increased trichome numbers above the wild type level (Payne *et al*., 2000). However, trichome clusters are found along the leaf margins of weak alleles of *ttg1*, indicating that in addition to its positive role in Arabidopsis trichome initiation, *TTG1* may also play a role in the lateral inhibition of neighboring cells from developing into trichomes (Larkin *et al*., 1999). A similar impact of *TTG1* on trichome clustering was observed when *BnTTG1* was knocked-down in the *B. napus* AtGL3^{+} background of line K-5-8. Trichomes on K-5-8 were not only taller and on more tissues, they were also present in more clusters compared with AtGL3^{+} plants. The increased clustering phenotype potentially may be due only to the low expression of *TRY* in K-5-8, since nests of trichomes have also been identified in Arabidopsis *try* mutants (Hulskamp *et al*., 1994). However, unlike Arabidopsis, manipulating *BnTTG1* levels in wild type *B. napus* did not result in a change to the wild type trichome phenotype, whereas in the AtGL3^{+} background it did. Hence, as with growth, *TTG1* may regulate trichome initiation and spacing through the *GL3* gene. The expression patterns of primary trichome regulatory genes in *B. napus* cv Westar plants over- or under- expressing *TTG1* needs to be carried out to garner additional support for such a hypothesis. Since more than 70 genes control trichome development in Arabidopsis, a comprehensive transcriptome analysis of *B. napus* cv Westar, AtGL3^{+}, K-5-8 and O-3-7 would also give a more comprehensive understanding of the role of *TTG1* in the context of other trichome development genes. Bimolecular complementation experiments and DNA binding studies would also allow for the identification of TTG1-related component protein MBW complexes in *B. napus*.

Based on expression patterns of the selected trichome development genes in this study, trichome production in *B. napus* appears to follow the two accepted theories for trichome development in Arabidopsis. For the activation of inhibition theory, tri-protein complexes and di-protein complexes activate the expression of both activators and inhibitors of trichome initiation in Arabidopsis (reviewed in Ishida *et al*., 2008), with the dosages of these primary regulators

determining trichome/non-trichome cell fate. Based on the *B. napus* data presented here and the findings in Arabidopsis that the presence of *TTG1* also prevents adjacent cells from developing into trichomes (Larkin *et al*., 1994), it appears that in *B. napus*, there has been a shift away from the inhibitory pathway towards the activator pathway in K-5-8 with a down-regulation of TTG1 levels and vice versa in O-3-7 with an up-regulation of TTG1 levels. According to the second, depletion theory (Bouyer *et al*., 2008; Balkunde *et al*., 2011), more GL3 which otherwise would bind to GL1 and activate the trichome initiation pathway, is titrated with the excess TTG1. Alternatively, since TTG1 can positively regulate R3MYBs (eg; TRY), GL3 may be depleted through binding with TRY (Morohashi and Grotewold, 2009).

The role of *TTG1* in regulating anthocyanin biosynthesis has also been studied extensively in Arabidopsis (reviewed in Petroni and Tonelli, 2011). Similar to trichome regulation, a tri-protein complex consisting of MYB-bHLH-WD40 proteins regulates anthocyanin accumulation (Baudry *et al*., 2006). As well as being involved in trichome development, *GL3*, a close homologue to *TT8*, is also involved in anthocyanin accumulation in leaves by positively regulating the structural genes *DFR* and *ANS* (Gonzalez *et al*., 2008). In this study, over-expression of *AtGL3* in *B. napus* cv Westar produced an increase in the expression of *DFR*, *ANS* and *GST* resulting in an increase in anthocyanin accumulation in seedling tissues. Similar to Arabidopsis (Gonzalez *et al*., 2008), in *B. napus*, the down regulation of *TTG1*, the WD40 factor in the MBW tri-protein complex, caused reduced expression of late biosynthesis genes *DFR*, *ANS* and *GST*, resulting in lower anthocyanin accumulation.

Understanding the trichome development regulatory pathway in glabrous *B. napus* is essential, as the reintroduction of this trait appears to have value for the development of stable resistance to insect pests. A trichome phenotype or stable resistance is especially important with the increasing number of pests and pathogens becoming resistant to chemical pesticides. However, transfer of resistance traits to cultivated species without compromising other vegetative and reproductive characteristics can be problematic. Genetic variation is important for a successful breeding program but alone is insufficient if inheritance of a trait is complex. In this thesis, enhanced trichome density and trichome length was achieved without compromising vegetative and reproductive growth by the manipulation of two genes in a very targeted way. These two genes are easily followed in successive generations and in this specific combination

(*AtGL3* expression and *BnTTG1* knock-down) represent the first breakthrough in the development of FB resistant cultivars in *B. napus* canola.

Whether the main problem with the endogenous *B. napus GL3* gene is due to sequence structure variation or transcript level still remains unclear. It is necessary as the next step to test whether the *AtGL3* provides an increase in a more appropriate transcript as a part of the understanding of why *B. napus* is normally glabrous. This is being addressed by next generation sequencing of trichome-dense and glabrous lines within the A, B, C and AC genomes of Brassica (Taheri, Nayidu and Gruber, unpublished). Sequence comparisons of *GL3* in these different lines and transformation of these variations into *B. napus* will help answer this question.

A comprehensive transcriptome analysis including patterns of co-expression, will highlight the target genes or partner genes for GL3 and TTG1. Each identified gene can also be tested in *B. napus* for enhanced trichome production and growth. However, transcript levels from each of those genes may not reflect their protein levels needed for optimum phenotypic expression. A study focusing on post-transcriptional and translational modification of target genes/proteins would be most helpful to gain a thorough understanding of trichome development and its relationship to plant growth and development in *B. napus*.

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6. APPENDIX

6.1 Primary trichome regulatory genes and their families

Gene	Gene Family
CPC	R3 MYB
CPL3	R3 MYB
EGL3	bHLH
ETC1	R3 MYB
ETC2	R3 MYB
GL1	R2R3 MYB
GL2	Homeodomain
GL3	bHLH
TCL1	R3 MYB
TCL2	R3 MYB
TRY	R3 MYB
TTG1	WD40
TTG2	WRKY

6.2 Alignments between Brassica sequences and Arabidopsis sequences, and primer coverage

TTG1

EF175930_*B. napus* isoform 1, **EF175932**_*B. napus* isoform 2, **HM208590.1**_*B. rapa*

```
EF175930     ATGGACAACTCAGCTCCAGACTCTTTACCTAGATCGGAAACCGCCGTCACCTACGACTCT 60
HM208590.1   ATGGACAACTCAGCTCCGGACTCCTTACCTAGATCGGAAACCGCCGTCACCTACGACTCT 60
EF175932     ATGGACAACTCAGCTCCGGACTCTTTACCTAGATCGGAAACCGCCGTCACCTACGACTCT 60
             ****************.***** **********************************

EF175930     CCTTACCCCCTCTACGCCATGTCCTTCTCCTCCTCCACCCACCGAATCGCCGTCGGGAGC 120
HM208590.1   CCTTACCCCCTCTACGCGATGTCCTTCTCCTCCTCCACCCACCGAATCGCCGTCGGAAGC 120
EF175932     CCGTACCCGCTCTACGCGATGTCCTTCTCCTCCTCCACCCACCGAATCGCCGTCGGGAGC 120
             ** ***** ******** ************************************.***

EF175930     TTCCTCGAGGACTACAACAACCGCATCGACATCCTCTCCTTCGACTCCGACTCCATGTCC 180
HM208590.1   TTCCTCGAAGACTACAACAACCGCATCGACATCCTCTCCTTCGACTCCGACTCCATGTCC 180
EF175932     TTCCTCGAGGACTACAACAACCGCATCGACATCCTCTCCTTCGACTCCGACTCCATGTCC 180
             ********.**************************************************

EF175930     CTCAAGCCCCTCCCGTCCCTCTCCTTCGAGCACCCTTACCCTCCCACCAAGCTCATGTTC 240
HM208590.1   CTCAAGCCCCTCCCGTCCCTCTCCTTCGAGCACCCTTACCCTCCCACCAAGCTCATGTTC 240
EF175932     CTCAAGCCCCTCCCATCCCTCTCCTTCGAGCACCCTTACCCTCCCACCAAGCTCATGTTC 240
             **************.*********************************************

EF175930     AGCCCCCCCTCCCTCCGCCGCAGCGGCGGCGGCGACCTCCTCGCCTCCTCCGGCGACTTC 300
HM208590.1   AGCCCCCCCTCCCTCCGCCGCAGCGGCGGCGGCGACCTCCTCGCCTCCTCCGGCGACTTC 300
EF175932     AGTCCCCCCTCCCTCCGCCGCAGCGGCGGGGGCGACCTCCTCGCCTCCTCCGGCGACTTC 300
             ** ************************** ******************************

EF175930     CTCCGCCTCTGGGAGGTCAACGAAGACTCCTCCTCCGCGGAGCCAGTATCCGTCCTCAAC 360
HM208590.1   CTCCGCCTCTGGGAGGTCAACGAAGACTCCTCCTCCGCGGAGCCAGTCTCCGTCCTCAAC 360
EF175932     CTCCGCCTCTGGGAGGTCAACGAAGACTCCTCCTCCGCGGAGCCAGTATCGGTCCTCAAC 360
             ********************************************.** *********

EF175930     AACAGCAAGACGAGCGAGTTCTGCGCGCCGCTGACCTCCTTCGACTGGAACGACGTCGAG 420
HM208590.1   AACAGCAAGACGAGCGAGTTCTGCGCGCCGCTGACCTCCTTCGACTGGAACGACGTCGAG 420
EF175932     AACAGCAAGACGAGCGAGTTCTGCGCGCCGCTGACCTCCTTCGACTGGAACGACGTGGAG 420
             ************************************************** ***

EF175930     CCGAAGCGGTTAGGCACGTGCAGCATCGACACCACGTGCACGATCTGGGACGTGGAGAGG 480
HM208590.1   CCGAAGCGGTTAGGCACGTGCAGCATCGACACCACGTGCACGATCTGGGACGTGGAGAGG 480
EF175932     CCGAAGCGGTTAGGCACGTGCAGCATCGACACCACGTGCACGATCTGGGACGTGGAGAGG 480
             ************************************************************

EF175930     TCCGTGGTGGAGACGCAGCTCATCGCGCACGACAAGGAGGTCCACGACATCGCGTGGGGG 540
HM208590.1   TCCGTGGTGGAGACGCAGCTCATCGCGCACGACAARGAGGTCCACGACATCGCGTGGGGG 540
EF175932     TCCGTGGTGGAGACGCAGCTCATCGCGCACGACAAGGAGGTCCACGACATCGCGTGGGGG 540
             ********************************** ************************

EF175930     GAGGCTAGGGTTTTCGCCTCGGTCTCCGCCGACGGATCGGTGAGGATCTTCGATCTGCGC 600
HM208590.1   GAGGCTAGGGTTTTCGCCTCGGTCTCCGCCGACGGATCGGTGAGGATCTTCGATCTGCGC 600
EF175932     GAGGCTAGGGTTTTCGCCTCGGTCTCCGCCGACGGATCGGTGAGGATCTTCGATCTGCGC 600
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EF175930    GACAAGGAGCACTCCACCATCATCTACGAGAGCCCCCAGCCCGATACGCCGCTCCTGAGG 660
HM208590.1  GACAAGGAGCACTCCACCATCATCTACGAGAGCCCCCAGCCCGATACGCCGCTCCTGAGG 660
EF175932    GACAAGGAGCACTCCACCATCATCTACGAGAGCCCCCAGCCCGATACGCCGCTCCTGAGG 660
            ************************************************************

EF175930    CTCGCGTGGAACAAGCAGGACTTGAGGTGTATGGCGACGATTCTGATGGATTCGAATAAG 720
HM208590.1  CTCGCCTGGAACAAGCAGGACTTGAGGTGTATGGCCACGATTCTGATGGATTCGAATAAG 720
EF175932    CTCGCGTGGAACAAGCAGGACTTGCGGTGTATGGCCACGATTCTGATGGATTCGAATAAG 720
            ***** ******************.********** ************************

EF175930    GTTGTGATTCTCGATATTCGATCGCCGACGATGCCTGTCGCGGAGCTGGAGCGGCACCAG 780
HM208590.1  GTTGTGATTCTCGATATTCGATCGCCGACGATGCCTGTCGCGGAGCTGGAGCGGCACCAG 780
EF175932    GTTGTCATTCTCGACATTCGATCGCCGACGATGCCGGTCGCCGAGCTTGAAAGGCACCAG 780
            ***** ******** ******************** ***** ***** **..******** 

EF175930    GGGAGTGTGAATGCGATTGCTTGGGCCCCGCAGAGCTGTAAGCATATCTGCTCGGGTGGG 840
HM208590.1  GGGAGTGTGAACGCGATTGCTTGGGCSCCGCAGAGCTGTAAGCATATCTGCTCGGGTGGK 840
EF175932    GGGAGTGTGAACGCGATTGCGTGGGCGCCGCAGAGCTGTAAGCATATCTGCTCGGGTGGG 840
            *********** ******** ***** ******************************** 

EF175930    GATGACGCGCAGGCTCTTATCTGGGAGTTGCCGACGATGGCTGGACCGAATGGGATTGAT 900
HM208590.1  GATGACGCGCAGGCTCTYATCTGGGAGTTGCCGACGATGGCTGGACCGAATGGGATTGAT 900
EF175932    GATGACGCGCAGGCTCTTATCTGGGAGTTGCCGACGATGGCTGGGCCGAATGGGATTGAT 900
            ***************** *************************.*************** 

EF175930    CCTATGTCGGTTTACTCGGCCGGTTCGGAGATTAACCAGCTGCAGTGGTCTTCTTCGTTG 960
HM208590.1  CCGATGTCGGTTTACTCGGCCGGTTCGGAGATTAACCAGCTGCAGTGGTCTTCTTCGTTG 960
EF175932    CCCATGTCGGTTTACTCGGCCGGTTCGGAGATTAACCAGTTGCAGTGGTCGGCTTCTCTG 960
            ** ********************************** **********  ****  ** 

EF175930    CCTGATTGGATTGGCATTGCGTTTGCTAACAAAATGCAGCTCCTTAGAGTTTGA 1014
HM208590.1  CCTGATTGGATTGGCATTGCGTTTGCTAACAAAATGCAGCTCCTTAGAGTTTGA 1014
EF175932    CCTGATTGGATTGGCATTGCGTTTGCTAACAAAATGCAGCTCCTCAGAGTTTGA 1014
            ****************************************** *********
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5’ - CG GGATCC ATGGACAACTCAGCTCCAGA - 3' – BamH1 (OFttg1)
5’ - CG GAGCTC TCAAACTCTAAGGAGCTGCA - 3' – Sac1 (ORttg1)

5’ - CG TCTAGA TAGGCACGTGCAGCATCGAC - 3' – Xba1 (LFttg1)
5’ - CG GGATCC ACACCTCAAGTCCTGCTTGT - 3’ – BamH1 (LRttg1)

5’ - CG GAGCTC TAGGCACGTGCAGCATCGAC - 3’ – Sac1 (RFttg1)
5’ - CG TACGTA ACACCTCAAGTCCTGCTTGT - 3’ – Snab1 (RRttg1)

5’ - CTCTGGGAGGTCAACGAA - 3’ – QLTTG1
5’ - ATGCTGCACGTGCCTAAC - 3’ – QRTTG1

GL3

GL3-F, **GL3-2**, **GL3-4**, **GL3-6** and **GL3-R** are different *BnGL3* clones, ATGL3 - **AT5G41315**

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GL3-F    -------------------------------------------------CGGGATATAAAG 12
GL3-2    ------------------------CAGCTTGGTCTACGGAGGAGCGAGCAGCTTAGAGAG 36
GL3-4    ------------------------CAGCTTGGTCTACGGAGGAGCGAGCAGCTTAGAGAG 36
GL3-6    ------------------------CAGCTTGGTCTACGGAGGAGCGAGCAGCTTAGAGAG 36
GL3-R    ------------------------CAGCTTGGTCTACGGAGGAGCGAGCAGCTTAGAGAG 36
ATGL3    CAAGCTTCGGAGATCAAAGCTGATCAGCTTGGTCTACGGAGGAGCGAGCAGCTTAGCGAG 240
                                                          * *  **   **

GL3-F    ACTATTGAGTCTCTCTCCATCGCTGA-TCTTCTTCCACTGGAACCGCCGCGGGATCTCAG 71
GL3-2    CTTTTTGAGTCTCTCTCCATCGCTGAATCTTCTTCCACTGGAACCGCCGCGGGATCTCAG 96
GL3-4    CTTTTTGAGTCTCTCTCCATCGCTGAATCTTCTTCCACTGGAACCGCCGCGGGATCTCAG 96
GL3-6    CTTTTTGAGTCTCTCTCCATCGCTGAATCTTCTTCCACTGGAACCGCCGCGGGATCTCAG 96
GL3-R    CTTTTTGAGTCTCTCTCCATCGCTGAATCTTCTTCCACTGGAACCGCCGCGGGATCTCAG 96
ATGL3    CTTTACGAGTCTCTCTCCGTCGCTGAATCTTCTTCTTCAGGCGTTGCTGCCGGATCTCAA 300
           *   ************ ******* ********  * **    ** ** ********

GL3-F    GTCAGTAGACGAGCTTCCGCGGCTGCACTCTCGCCGGAAGATATCGCCGACACCGAGTGG 131
GL3-2    GTCAGTAGACGAGCTTCCGCGGCTGCACTCTCGCCGGAAGATCTCGCCGACACCGAGTGG 156
GL3-4    GTCAGTAGACGAGCTTCCGCGGCTGCACTCTCGCCGGAGGATCTCGCCGACACCGAGTGG 156
GL3-6    GTCAGTAGACGAGCTTCCGCGGCTGCACTCTCGCCGGAAGATCTCGCCGACACCGAGTGG 156
GL3-R    GTCAGTAGACGAGCTTCCGCGGCTGCACTCTCGCCGGAAGATCTCGCCGACACCGAGTGG 156
ATGL3    GTCACCAGACGAGCTTCCGCCGCCGCACTTTCACCGGAAGATCTCGCCGACACCGAGTGG 360
         ****  ************** ** ***** ** ***** *** ****************

GL3      TACTACTTGGTTTGTATGTCTTTCGTCTTCAAAATCGGTGAAGGAATGCCTGGACGGACT 191
GL3      TACTACTTGGTTTGTATGTCTTTCGTCTTCAAAATCGGTGAAGGAATGCCTGGACGGACT 216
GL3      TACTACTTGGTTTGTATGTCTTTCGTCTTCAAAATCGGTGAAGGAATGCCTGGACGGACT 216
GL3      TACTACTTGGTTTGTATGTCTTTCGTCTTCAAAATCGGTGAAGGAATGCCTGGACGGACT 216
GL3      TACTACTTGGTTTGTATGTCTTTCGTCTTCAAAATCGGTGAAGGAATGCCTGGACGGACT 216
ATGL3    TACTATTTGGTTTGTATGTCTTTCGTCTTCAACATTGGTGAAGGAATGCCTGGACGGACG 420
         ***** ************************** ** ***********************

GL3-F    TTTGCAAACGGTGAACCGATATGGCTATGCAATGCTGGTACTGCTGATAGTAAAGTTTTT 251
GL3-2    TTTGCAAACGGTGAACCGATATGGCTATGCAATGCTGGTACTGCTGATAGTAAAGTTTTT 276
GL3-4    TTTGCAAACGGTGAACCGATATGGCTATGCAATGCTGGTACTGCTGATAGTAAAGTTTTT 276
GL3-6    TTTGCAAACGGTGAACCGATATGGCTATGCAATGCTGGTACTGCTGATAGTAAAGTTTTT 276
GL3-R    TTTGCAAACGGTGAACCGATATGGCTATGCAATGCTGGTACTGCTGATAGTAAAGTTTTT 276
ATGL3    TTTGCAAACGGTGAACCGATATGGTTGTGCAACGCTCATACGGCGGATAGTAAAGTGTTT 480
         ********************** * ***** ***  *** ** *********** ***

GL3-F    AGCCGTTCTCTCCTCGCTAAAAGCGCTTCGGTTAATACAGTAATTTGCTTCCCGTTTCTT 311
GL3-2    AGCCGTTCTCTCCTCGCTAAAAGCGCTTCGGTTAATACAGTAATTTGCTTCCCGTTTCTT 336
GL3-4    AGCCGTTCTCTCCTCGCTAAAAGCGCTTCGGTTAATACAGTAATTTGCTTCCCGTTTCTT 336
GL3-6    AGCCGTTCTCTCCTCGCTAAAAGCGCTTCGGTTAATACAGTAATTTGCTTCCCGTTTCTT 336
GL3-R    AGCCGTTCTCTCCTCGCTAAAAGCGCTTCGGTTAATACAGTAATTTGCTTCCCGTTTCTT 336
ATGL3    AGCCGTTCTCTTCTAGCAAAAAGTGCTGCGGTTAAGACAGTGGTTTGCTTCCCGTTCCTT 540
         *********** ** ** ***** *** ******* *****  ************* ***
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GL3-F    GGAGGAGTCGTTGAGATCGGTACCACAGAACACATTGCAGAGGATATGAACGTAATACAA 371
GL3-2    GGAGGAGTCGTTGAGATCGGTACCACAGAACACATTGCAGAGGATATGAACGTAATACAA 396
GL3-4    GGAGGAGTCGTTGAGATCGGTACCACAGAACACATTGCAGAGGATATGAACGTAATACAA 396
GL3-6    GGAGGAGTCGTTGAGATCGGTACCACAGAACACATTGCAGAGGATATGAACGTAATACAA 396
GL3-R    GGAGGAGTCGTTGAGATCGGTACCACAGAACACATTGCAGAGGATATGAACGTAATACAA 396
ATGL3    GGAGGAGTCGTTGAGATTGGTACCACAGAACATATTACGGAAGACATGAATGTAATACAA 600
         ********************* ************** *** * ** ** ***** *********

GL3-F    TGCGTGAAGAAATCATTCCTCGAAGCTCCTGATTCAAACGCTACAATATTACAACCAATA 431
GL3-2    TGCGTGAAGAAATCATTCCTCGAAGCTCCTGATTCAAACGCTACAATATTACAACCAATA 456
GL3-4    TGCGTGAAGAAATCATTCCTCGAAGCTCCTGATTCAAACGCTACAATATTACAACCAATA 456
GL3-6    TGCGTGAAGAAATCATTCCTCGAAGCTCCTGATTCAAACGCTACAATATTACAACCAATA 456
GL3-R    TGCGTGAAGAAATCATTCCTCGAAGCTCCTGATTCAAACGCTACAATATTACAACCAATA 456
ATGL3    TGCGTGAAGACATCATTCCTCGAAGCCCCTGATCCGTACGCTACAATATTACCAGCAAGA 660
         ********** *************** ****** *  *************** * *** *

GL3-F    TCATCAGATTATCACATAGACAACGTCCTCGATCCGCAACACATCCTAGAAGACGAAATC 491
GL3-2    TCATCAGATTATCACATAGACAACGTCCTCGATCCGCAACACATCCTAGAAGACGAAATC 516
GL3-4    TCATCAGATTATCACATAGACAACGTCCTCGATCCGCAACACATCCTAGAAGACGAAATC 516
GL3-6    TCATCAGATTATCACATAGACAACGTCCTCGATCCGCAACACATCCTAGAAGACGAAATC 516
GL3-R    TCATCAGATTATCACATAGACAACGTCCTCGATCCGCAACACATCCTAGAAGACGAAATC 516
ATGL3    TCC---GATTATCACATCGACAACGTTCTTGATCCGCAACAGATTCTAGGCGACGAGATT 717
         **    *********** ******** ** *********** ** ****  ***** **

GL3-F    TATGCTCCTATGTTTGGTACTCGGCCTTTTCAAGCAGCTTCTCCGATCAGAACTCA---- 547
GL3-2    TATGCTCCTATGTTTGGTACTCGGCCTTTTCAAGCAGCTTCTCCGAGCAGAACT------ 570
GL3-4    TATGCTCCTATGTTTGGTACTCGGCCTTTTCAAGCAGCTTCTCCGAGCAGAACT------ 570
GL3-6    TATGCTCCTATGTTTGGTACTCGGCCTTTTCAAGCAGCTTCTCCGAGCAGAACT------ 570
GL3-R    TATGCTCCTAT--------TTCAGTTTTGTTAAATCGG---------------------- 546
ATGL3    TACGCGCCTATGTTCAGTACGGAGCCTTTTCCAACAGCTTCTCCGAGCAGAACTACCAAC 777
         ** ** *****           *  ** *  *   *
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5’ - GGCTATGCAATGCTGGTACT - 3’ – QLGL3
5’ - CCTCTGCAATGTGTTCTGTG - 3’ – QRGL3

5’ - ACTTTCACCGGAAGATCTCG - 3’ – QLAtGL3
5’ - TTACTATCCGCCGTATGAGC - 3’ – QRAtGL3

GL1

BN_GL1 - **HQ162473** (*B. napus*), BI_GL1 - **HQ162472** (*B. incana*), BO_GL1 - **HQ162471** (*B. oleracea*) and BR_GL1 - **HQ162470** (*B. rapa*)

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BN_GL1   ATGAGAACGAGGAGAAGAACAGAGGAAGAGAATCATCAAGAATACAAGAAAGGGTTGTGG 60
BI_GL1   ATGAGAACGAGGAGAAGAACAGAGGAAGAGAATCATCAAGAATACAAGAAAGGGTTGTGG 60
BO_GL1   ATGAGAACGAGGAGAAGAACAGAGGAAGAGAATCATCAAGAATACAAGAAAGGGTTGTGG 60
BR_GL1   ATGAGAACGAGGAGAAGAACAGAGGAAGAGAATCATCAAGAATACAAGAAAGGGTTGTGG 60
         ************************************************************

BN_GL1   ACAGTTGAAGAAGACAACATTCTTAGGGACCATGTCCTTACTCACGGCAAAGGCCAATGG 120
BI_GL1   ACAGTTGAAGAAGACAACATTCTTAGGGACCATGTCCTTACTTACGGCAAAGGCCAATGG 120
BO_GL1   ACAGTTGAAGAAGACAGCATTCTTAGGGACTATGTCCTTACTCACGGCAAAGGCCAATGG 120
BR_GL1   ACAGTTGAAGAAGACAACATTCTTAGGGACTATGTCCTTACTCACGGCAAAGGCCAATGG 120
         *************** ************* *********** *****************

BN_GL1   AACCGCATCGTCAGGAAAACTGGGCTGAAGAGGTGTGGAAAGAGCTGTAGACTTAGATGG 180
BI_GL1   AACCGTATCGTCAGGAAAACTGGGCTGAAGAGGTGTGGAAAGAGCTGTAGACTTAGATGG 180
BO_GL1   AACCGCATCGTCAGGAAAACTGGGCTGAAGAGGTGTGGAAAGAGCTGTAGACTTAGATGG 180
BR_GL1   AACCGCATCGTCAGGAAAACTGGGCTGAAGAGGTGTGGAAAGAGCTGTAGACTTAGATGG 180
         ***** ******************************************************

BN_GL1   ATAAATTATCTGAGCCCTAATGTGAATAAAGGTAATTTCACTGAACAAGAAGAAGACCTC 240
BI_GL1   ATAAATTATCTGAGCCCTAATGTGAATAAAGGTAATTTCACTGAACAAGAAGAAGACCTC 240
BO_GL1   ATAAATTATCTGAGCCCTAATGTGAATAAAGGTAATTTCACTGAACAAGAAGAAGACCTC 240
BR_GL1   ATAAATTATCTGAGCCCTAATGTGAATAAAGGTAATTTCACTGAACAAGAAGAAGACCTC 240
         ************************************************************

BN_GL1   ATTATTCGTCTCCACAAGCTACTTGGCAACAGATGGTCTTTAATAGCTAAAAGGGTACCT 300
BI_GL1   ATTATTCGTCTCCACAAGCTACTTGGCAACAGATGGTCTTTAATAGCTAAAAGGGTACCT 300
BO_GL1   ATTATTCGTCTCCACAAGCTACTTGGCAACAGATGGTCTTTAATAGCTAAAAGGGTACCT 300
BR_GL1   ATTATTCGTCTCCACAAGCTACTTGGCAACAGATGGTCTTTAATAGCTAAAAGGGTACCT 300
         ************************************************************

BN_GL1   GGAAGGACAGATAACCAAGTCAAGAATCACTGGAACACTCATCTCAGCAAAAAAA-TCGT 359
BI_GL1   GGAAGGACAGATAACCAAGTCAAGAATCACTGGAACACTCATCTCAGCAAAAAAA-TCGT 359
BO_GL1   GGAAGGACAGATAACCAAGTCAAGAATCACTGGAGCACTCATCTCAGCAAAAAAAATCGT 360
BR_GL1   GGAAGGACAGATAACCAAGTCAAGAATCACTGGAACACTCATCTCAGCAAAAAAA-TCGT 359
         ********************************** ****************** ****

BN_GL1   CGGGGATTATTCCTCTGCTGTCAAAACCACTGGAGAAGAAAACTATCCACCGTCACTACT 419
BI_GL1   CGGGGATTATTCCTCTGCTGTCAAAACCACTGGAGAAGAAAACTATCCACCGTCACTACT 419
BO_GL1   CGGGGATTAT-CCTCTGCTGTCAAAACTACTGGAGAAGAAAACTATCCACCGTCACTACT 419
BR_GL1   CGGGGATTATTCCTCCGCTGTCAAAACCACTGGAGAAGAAAACGATCCACCGTCACTACT 419
         ********** **** ********** *************** ****************

BN_GL1   CATCACCGCCGCAACAGCTTCTGGTCATCATCAACAAGACAAAATCTGTGACAAGAGTTT 479
BI_GL1   CATCACCGCCGCAACAGCTTCTGGTCATCATCAACAAGACAAAATCTGTGACAAGAGTTT 479
BO_GL1   CATCACCGCCGCAACAGCTTCTGGTCATCATCAACAAGACAAAATCTGTGACAAGAGTTT 479
BR_GL1   CATCACCGCCGCAACAACTTCTGGTCATCATCAACAAGACAAAATCTGTGACAAGAGTTT 479
         *************** ******************************************
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BN_GL1   CGAGGGCCTTGTATCAGCTTCGTACGGGAATAAACAAAAAGCGGATTTGACACATACTAA 539
BI_GL1   CGAGGGCCTTGTATCAGCTTCGTACGGGAATAAACAAAAAGCGGATTTGACACATACTAA 539
BO_GL1   CGAGGGCCTTGTATCAGCTTCGAACGGGAATAAACAAAAAGCGGATTTGACACATACTAA 539
BR_GL1   CGAGGGCCTCGTATCTGCTTCGTACGAAAATAAACAAAAAGCGGATTTGACACATACTAA 539
         ********* ***** ****** ***  ******************************

BN_GL1   TGATTTGAGTCTCTACTTCAAAGAGAGGAACAACTTTGATAGCAGTAACGCTTTCTGGTT 599
BI_GL1   TGATTTGAGTCTCTACTTCAAAGAGAGGAACAACTTTGATAGCAGTAACGCTTTCTGGTT 599
BO_GL1   TGATTTGAGTCTCTACTTCAAAGAGAGGAACAACTTTGATAGCAGTAACACTTTCTGGTT 599
BR_GL1   TGATTCGAGTCTCTACTTCAAAGAGAGGAACAACTTTGATAGCAGTAACGCTTTCTGGTT 599
         ***** ****************************************** **********

BN_GL1   TAATGACGCCGATGATTTTGAGATGAATTCATCGGCTATGATGGATTTTGCGTCGGGTGA 659
BI_GL1   TAATGACGCCGATGATTTTGAGATGAATTCATTCGCTATGATGGATTTTGCGTCGGGTGA 659
BO_GL1   TAATGACGACGATGATTTTGAGATGAATC----------------------------- 628
BR_GL1   TAATGACGACGATGATTTTGAGATGAATTCATTCGCTATGATGGATTTTGCGTCCGGTGA 659
         ******** ******************

BN_GL1   TACTGGCTACTGTCTCTAG 678
BI_GL1   TACTGGCTACTGTCTCTAG 678
BO_GL1   ------------------
BR_GL1   TACTGGCTACTGCCTCTAG 678
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5’ - CCACAAGCTACTTGGCAACA - 3’ – QLGL1
5’ - CCAGTGGTTTTGACAGCAGA - 3’ – QRGL1

GL2

EU826520 - *BnGL2*a, **EU826521 -** *BnGL2*b

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EU826520  ATGTCAATGGCCGTCGAGATGTCATCGAAACAACCCACCAAAGACTTCTTCTCTTCTCCA 60
EU826521  ATGTCAATGGCCGTCGAGATGTCATCGAAACAACCCACCAAAGACTTCTTCTCCTCTCCA 60
          ************************************************** ******

EU826520  GCCCTCTCCCTCTCTCTCGCTGGGATATTTCGGAATGCATCCTCCGGCAGCACGAACCCT 120
EU826521  GCCCTCTCCCTCTCTCTCGCTGGGATATTTCGGAATGCATCCTCCGGCAGCACGAACCCT 120
          ************************************************************

EU826520  GAGGAGGACTTTCTGGGAAGAAGAGTAGCTGACGATGAGGATCGGACGGTGGAGATGAGC 180
EU826521  GAGGAGGACTTTCTGGGAAGAAGAGTAGCTGACGATGAGGATCGGACGGTGGAGATGAGC 180
          ************************************************************

EU826520  AGCGAGAACTCGGGACCCACGAGATCCAGATCAAAGGACAATTTGGAGGGTGAGGAGGAT 240
EU826521  AGCGAGAACTCGGGACCCACGAGATCCAGATCAGAGGACAATTTGGAGGGTGAGGAGGAT 240
          ********************************* ************************

EU826520  CAAGAGGAAGAGGATGGTGCAGGAAACAAGGGCAACAAGAGGAAGAGGAAGAAGTATCAT 300
EU826521  CAAGAGGAAGAGGATGGTGCAGGAAACAAGGGCAACAAGAGGAAGAGGAAGAAGTATCAT 300
          ************************************************************

EU826520  CGCCACACCACCGATCAAATTAGACACATGGAAGCGCTGTTCAAAGAAACACCTCATCCG 360
EU826521  CGCCACACCACCGATCAGATTAGACACATGGAAGCGCTGTTCAAAGAAACACCTCATCCG 360
          ***************** ****************************************

EU826520  GACGAGAAGCAAAGACAGCAGCTGAGCAAGCAATTAGGACTAGCTCCTCGCCAAGTCAAG 420
EU826521  GACGAGAAGCAAAGACAGCAGCTGAGCAAGCAATTAGGACTAGCTCCTCGCCAAGTCAAG 420
          ************************************************************

EU826520  TTCTGGTTCCAAAACCGCCGCACCCAGATCAAGGCTATTCAAGAACGGCATGAAAACTCA 480
EU826521  TTCTGGTTCCAAAACCGCCGCACCCAGATCAAGGCTATTCAAGAACGGCATGAAAACTCA 480
          ************************************************************

EU826520  TTGCTGAAAGCGGAGCTAGAGAAGCTGAGAGAGGAAAACAAAGCGATGAGAGAGTCATTT 540
EU826521  TTGCTGAAAGCGGAGCTAGAGAAGCTGAGAGAGGAAAACAAAGCGATGAGAGAGTCATTT 540
          ************************************************************

EU826520  TCCAAGGCTAATTCCTCCTCGTGCCCCAACTGCGGAGGAGGAGGAGGAGGAGGAGGAAGC 600
EU826521  TCCAAGGCTAATTCCTCCTCGTGCCCCAACTGCGGAGGAGGAGGAGGAGGAGGAGGAAGC 600
          ************************************************************

EU826520  CCCGATGATCTCCACCTTGAAAACACCAAACTAAAAGCCGAGCTCGACAAGCTTCGGGCG 660
EU826521  CCCGATGATCTCCACCTTGAAAACACCAAACTAAAAGCCGAGCTCGACAAGCTTCGTGCG 660
          ********************************************************** ***

EU826520  GCTCTCGGACGGACTCCCTATCCCCTTCAGGCCTCATGCTCCGATGATCAACAGCGCCGT 720
EU826521  GCTCTCGGACGGACTCCCTATCCCCTTCAGGCCTCATGCTCCGATGATCAACATCGACGT 720
          ********************************************** ** ***

EU826520  GTTGGCTCACTCGAACTCTACACGGGGGTCTTTGCCCTCGAGAAGTCCCGCATCGTGGAG 780
EU826521  GTTGGCTCCCTAGAACTCTACACGGGGGTCTTTGCCCTCGAGAAGTCCCGCATTGTGGAG 780
          ******** ** ****************************************** ******
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EU826520  ATTGCCAACCGAGCCACCCTTGAACTTCAGAAGATGGCCACCTCCGGTGAACCTCTGTGG 840
EU826521  ATTGCCAACCGAGCCACCCTCGAAGTTCAGAAGATGGCCACATCCGGCGAACCTCTATGG 840
          ******************** *** *************** ***** ******** ***

EU826520  CTCCGAAGCCTTGAGACTGGCCGTGAGATTCTCAACTACGATGAGTATCTCAAGGAGTTT 900
EU826521  CTCCGAAGCCTTGAGACTGGCCGTGAGATTCTCAACTACGATGAGTATCTCAAGGAGTTT 900
          ************************************************************

EU826520  CCTCAAGCTCAGGCCTCTTCATTTCATGGGAGAAAAACCATTGAAGCCTCCCGAGATGTG 960
EU826521  CCTCAAGCTCAGGCCTCTTCATTTCATGGGAGGAAAACCATTGAAGCATCCCGAGATGTG 960
          ******************************** ************* ************

EU826520  GGGATCGTGTTTATGGACGCACATAAACTTGCCCAAAGTTTCATGGACGTGGAGCAATGG 1020
EU826521  GGGATCGTGTTTATGGACGCACATAAACTTGCCCAAAGTTTCATGGACGTGGAGCAATGG 1020
          ************************************************************

EU826520  AAAGAGATGTTTGCATGTTTGATCTCAAAGGCGGTTACGGTTGATGTAATTCGACAGGGT 1080
EU826521  AAAGAGATGTTTGCATGTTTGATCTCAAAGGCGGTGACGGTTGATGTAATTCGACAGGGT 1080
          ********************************** ************************

EU826520  GAAGGGCCTTCCAGGATCGACGGTGCCATTCAGTTGATGTTTGGGGAGATGCAGCTGCTC 1140
EU826521  GAAGGGCCTTCAAGGATCGACGGTGCCATTCAATTGATGTTTGGGGAGATGCAGCTGCTC 1140
          *********** ******************** ****************************

EU826520  ACTCCAGTTGTCCCAACAAGAGAAGTCTACTTTGTGAGAAGTTGCCGCCAGCTGAGCCCT 1200
EU826521  ACTCCAGTTGTCCCCACAAGAGAAGTCTACTTTGTGAGAAGTTGCCGGCAGCTGAGTCCC 1200
          ************** ********************************* ******** **

EU826520  GAGAAATGGGCCATCGTGGATGTATCGGTCTCTATGGAGGAGGACAACAACG---CAGAG 1257
EU826521  GAGAAATGGGTCATCGTGGATGTATCTGTTTCTGTGGAGGAGGACAACAACAGTACAGAG 1260
          ********** *************** ** *** *****************    *****

EU826520  AAGGAGGGTTCACTGCTGAGATGTCGGAAGCGCCCTTCAGGGTGCATCATCGAGGACACC 1317
EU826521  AAGGAGGGTTCACTGCTGAGATGTCGGAAGCGCCCTTCAGGGTGCATTATAGAGGACACC 1320
          ********************************************** ** *********

EU826520  TCCAACGGCCACTCCAAGGTCACTTGGGTGGAGCATCTTGACTTGTCTGCTTCCACGGTT 1377
EU826521  TCCAACGGCCATTCCAAGGTCACCTGGGTGGAGCACCTTGACTTGTCTGCTTCCACAGTT 1380
          *********** *********** *********** ******************** ***

EU826520  CAGCCTCTCTTCCGCTCTTTTGTCAACACCGGTTTGGCCTTTGGTGCTAAACATTGGGTC 1437
EU826521  CAGCCTCTCTTCCGCTCCTTTGTCAACACCGGTTTGGCCTTTGGTGCTCGTCATTGGGTT 1440
          ***************** ******************************   ********

EU826520  GCCACCCTTCAGCTCCACTGCGAACGACTTGTCTTCTTCATGGCTACCAACGTCCCCACC 1497
EU826521  GCCACCCTTCAGCTCCACTGCGAACGACTTGTCTTCTTCATGGCTACCAACGTCCCCACC 1500
          ************************************************************

EU826520  AAGGACTCTCTCGGGGTTACAACGCTGGCCGGGAGAAAGAGCGTACTCAAGATGGCGCAG 1557
EU826521  AAGGACTCTCTCGGAGTTACAACGCTGGCCGGGAGAAAGAGCGTACTCAAGATGGCGCAG 1560
          ************** ********************************************

EU826520  AGGATGACACAAAGTTTCTACCGCGCCATTGCTGCTTCTAGCTACCATCAGTGGAACAAG 1617
EU826521  AGGATGACACAAAGCTTCTACCGCGCCATTGCTGCTTCTAGCTACCATCAGTGGAACAAG 1620
          ************** ********************************************
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EU826520  ATCACCACCAAAACTGGACAAGACATGAGGGTTTCATCCAGAAAAAACCTCCATGATCCT 1677
EU826521  ATCACCACCAAAACTGGACAAGACATGAGGGTTTCATCCAGAAAAAACCTCCATGATCCT 1680
          ************************************************************

EU826520  GGAGAGCCTACCGGAGTCATAGTTTGCGCTTCTTCCTCCCTCTGGTTACCTGTTTCTCCT 1737
EU826521  GGCGAGCCTACCGGAGTCATAGTTTGCGCTTCTTCCTCCCTCTGGTTACCTGTTTCTCCT 1740
          ** ********************************************************

EU826520  ACTCTTCTCTTTGATTTCTTTAGAGATGAAACTCGTCGCCATGAGTGGGATGCTTTGTCA 1797
EU826521  ACTCTTCTCTTTGATTTCTTTAGAGATGAAACTCGTCGCCATGAGTGGGATGCTTTGTCA 1800
          ************************************************************

EU826520  AACGGGGCTCATGTTCAGTCAATTGTAAGCTTATCCAAGGGACAAGATAGAGGCAACTCA 1857
EU826521  AACGGAGCTCATGTTCAGTCTATTGTAAGCTTATCCAAGGGACAAGACAGGGGCAACTCC 1860
          ***** ************** ************************** ** ********

EU826520  GTGTCTATCCAGACAGTGAAATCGAGAGAAAAGAGCACATGGGTGCTGCAAGACAGCTGC 1917
EU826521  GTGTCTATCCAGACAGTGAAATCGAGAGAAAAGAGCACATGGGTGCTGCAGGACAGCTGC 1920
          ************************************************* *********

EU826520  ACAAACTCATATGAGTCGGTGGTTGTATACGCTCCCGTAGATATAAACACGACACAGATG 1977
EU826521  ACAAACTCATATGAGTCGGTGGTTGTATACGCTCCTGTAGATATCAACACGACACAGCTC 1980
          ********************************** ******** ************ *

EU826520  GTGATCGCAGGACATGATCCAAGCAACATCCAAATCCTCCCTTGTGGATTCTCAATCATA 2037
EU826521  GTGATTGCCGGACATGATCCAAGCAACATCCAAATCCTCCCTTGTGGATTCTCAATCATA 2040
          ***** ** ************************************************

EU826520  CCTGATGGAGTAGAATCACGACAACTGGTAATCTCGTCTGCACAAGAGGCAGACAGAAAC 2097
EU826521  CCTGATGGAGTAGAATCACGACAATTGGTCATCTCGTCTGCACAAGAGG---ACAGAAAC 2097
          ************************ **** ******************   ********

EU826520  ACACAAGGAGGGTCTCTGCTCACAATGGCCCTCCAAACGCTCGTCAACCAATCTCCGGCA 2157
EU826521  ACACAAGGAGGGTCTCTGCTCACACTGGCCCTCCAAACGCTCGTCAACCAATCTCCTGCA 2157
          ************************ ******************************** ***

EU826520  GCAAAGCTGAATATGGAGTCTGTGGAATCAGTCACAAACCTCGTCTCTGTCACGCTGCAC 2217
EU826521  GCAAAGCTGAATATGGAGTCTGTGGAATCCGTTACAAACCTCGTCTCTGTCACGCTCCAC 2217
          ***************************** ** ********************** ***

EU826520  AACATTAAGAGATGCCTACAAATCGAAGATTGTTGA 2253
EU826521  AACATTAAGAGAAGCCTACAAATCGAAGATTGTTGA 2253
          ************ ************************
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5’ – CGCTGGCCGGGAGAAAGAGC - 3’ – QLGL2
5’ - GGAGGTTTTTTCTGGATGAA - 3’ – QRGL2

TRY

EE451172- *BnTRY*, **AT5G53200–** *AtTRY*

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EE451172    ATGGATAACACTGACCGTCGTCGCCGTCGTAAGCAACACAAAGTCACTCTCCATGACTCT 60
AT5G53200   ATGGATAACACTGACCGTCGTCGCCGTCGTAAGCAACACAAAATCGCCCTCCATGACTCT 60
            ****************************************.**.* ************

EE451172    GAAGAAGTGAGCAGTATTGAATGGGAGTTTATCAATATGACAGAACAAGAAGAAGATCTC 120
AT5G53200   GAAGAAGTGAGCAGTATCGAATGGGAGTTTATCAACATGACTGAACAAGAAGAAGATCTC 120
            ***************** ***************** ******:******************

EE451172    ATCTTTCGAATGCATAGACTTGTCGGTGATAGGTGGGATTTAATAGCAGGACGAGTGCCA 180
AT5G53200   ATCTTTCGAATGTACAGACTTGTCGGTGATAGGTGGGATTTGATAGCAGGAAGAGTTCCT 180
            ************ * *************************.*********.**** **:

EE451172    GGAAGACAACCAGAAGAGATAGAGAGATACTGGATAATGAGAAACAGTGATGGCTTTGCT 240
AT5G53200   GGAAGACAACCAGAGGAGATAGAGAGATATTGGATAATGAGAAACAGTGAAGGCTTTGCT 240
            **************.************** ********************:*********

EE451172    GAGAAACGACGCCAACTTCATCACTCCTCTTCTCACAAAAGTACCAAACCTCATCGTCCA 300
AT5G53200   GATAAACGACGCCAGCT---TCACTCATCTTCCCACAAACATACCAAGCCTCACCGTCCT 297
            ** ************.**   ******.***** ******..******.***** *****:

EE451172    CGTTTTTCTATTTATCCTTCTTAG 324
AT5G53200   CGCTTTTCTATCTATCCTTCCTAG 321
            ** ******** ******** ***
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5' - TCGGTGATAGGTGGGATTTA - 3' – QLTRY
5' - GTGGACGATGAGGTTTGGTA - 3' – QRTRY

6.3 LIST OF BUFFERS

50x TAE buffer (Tri-acetate) – per litre

2 M Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA, pH 8.0

2x CTAB buffer

2% (w/v) cetyl-trimethyl-ammonium bromide, 1.4 M NaCl, 100 mM Tris HCl (pH 8.0), 20 mM EDTA

Southern hybridization buffer

1% BSA, 7% SDS, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2

20x SSC buffer

3 M NaCl, 0.3 M sodium citrate, pH 7.0 with 10 N NaOH

6.4 LIST OF MEDIA

Coco mix

4 x 5 kg soaked and shredded co-co fibre

1 x 107 L bale peat moss

3.2 kg fine ground Calcium Carbonate

4 kg 15-9-12 “Osmocote PLUS” controlled release fertilizer with minor nutrients

400 g Superphosphate

15 g “Fritted” Trace Elements # 503

15 g 13% chelated iron

7 g 14% chelated zinc

Washed “Torpedo” sand, screened to 1 cm maximum particle size

Soiless mix

1 x 107 L bale peat moss

3.2 kg fine ground Calcium Carbonate

4 kg 15-9-12 “Osmocote PLUS” controlled release fertilizer with minor nutrients

400 g Superphosphate

15 g “Fritted” Trace Elements # 503

15 g 13% chelated iron

7 g 14% chelated zinc

Washed “Torpedo” sand, screened to 1 cm maximum particle size

LB medium - 1% NaCl, 1% Tryptone, 0.5% Yeast extract, 1.5% Bacto agar

Co-cultivation medium - MS, 0.5g/L MES (pH5.8), 0.125mg/L NAA, 0.5mg/L BAP, 0.5% agarose

Hypo I medium - MS, 0.5 g/l MES, 40 mg/L adenine-SO_4, 0.5 g/l PVP, 0.5% agar, 0.5 mg/L NAA, 1 mg/L BAP, 5 mg/L $AgNO_3$, 5 mg/L L-ppt, 12 g/L glucose, 450 mg/L timentin, pH5.8

Hypo II medium - MS, 0.5 g/L MES, 40 mg/L adenine-SO_4, 0.5 g/L PVP, 0.6% agar, 1.0 mg/L BAP, 0.5 mg/L NAA, 20 g/L sucrose, 5 mg/L L-ppt, 450 mg/L timentin, pH5.8

Hypo III medium - MS, 0.5 g/L MES, 40 mg/L adenine-SO_4, 0.5 g/L PVP, 0.6% agar, 1.0 mg/L BAP, 20 g/L sucrose, 5 mg/L L-ppt, 450 mg/L timentin, pH5.8

Hypo IV medium - MS, 3% sucrose, 0.1 mg/L NAA, 0.7% agar, pH5.8